

**Mechanisms of starch degradation in turions
of
*Spirodela polyrhiza***

Doctoral dissertation

submitted by

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Gutachter:

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Tag des Rigorosums:

Tag der öffentlichen Verteidigung:

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AbbreviationsGeneral abbreviations in common use, chemicals and enzymes

ATP	Adenosine triphosphate
BSA	Albumine Bovine Serum
CIAP	Calf intestine alkaline phosphatase
cR	Continuous red light
DTT	Dithiothreitol
1D-E	One dimensional electrophoresis
2D-E	Two dimensional electrophoresis
EDTA	Ethylendiamintetraacetat
FPLC	Fast Performance Liquid Chromatography
HEPES	N-2-Hydroxyethylpiperazin-N`-2-ethanolsulfon acid
HCl	Hydrochloric acid
HPAEC-PAD	High Performance Anion Exchange Chromatography -Amperometric Detection
GWD	Glucan-water-dikinase
nc-AFM	non-Contact Atomic Force Microscopy
NaOH	Sodium hydroxyde
PAGE	Polyacrilamid gel electrophoresis
PIPES	Piperazin-1,4-N-2-ethanolsulfon acid
PVDF	Polyvinylidene-difluoride
Rp	Single red pulse
SDS	Sodiumdodecylsulphat
SEM	Scanning Electron Microscopy
Tris	Tris-(hydroxymethyl)-aminomethan

Measuring units

°C	Grad Celsius
C	Curie

cpm	Counts per minute
DW	Dry weight
µg	Microgramme
µl	Microlitre
µM	Micromolar
FW	Fresh weight
g	Gramme
h	Hour
kDa	Kilodalton
kVh	Kilovolt per hour
λ_{\max}	Wave length with the highest transmission rate
M	Mol
mA	Milliamper
min	Minute
ml	Millilitre
mM	Millimolar
OD	Optical densisty
rpm	Rotations per minute
s	Second
U	Enzymatic unit
vol	Volume
v/v	Volume pro volume
w/v	Weight pro volume
w/W	Weight pro weight

Organisms

<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>P. sativum</i>	<i>Pisum sativum</i>
<i>S. polyrhiza</i>	<i>Spirodela polyrhiza</i>
<i>S. tuberosum</i>	<i>Solanum tuberosum</i>

1. INTRODUCTION

1.1. Starch structure and function

Starch is the major energy storage compound in higher plants. The name starch represents semicrystalline particles composed exclusively of glucose residues. The glucose moieties are linked by only two types of bonds: α -1,4 and α -1,6 glucosidic linkages. The main constituents of starch are amylopectine (~75% by weight), and amylose (~25% by weight) (Blennow *et al.*, 2002). Amylopectine is a semicrystalline, highly branched polysaccharide with an α -1,4 backbone and 4-5% α -1,6 branch points (Ball *et al.*, 1998). The degree of polymerisation of amylopectine is 10^3 - 10^4 . Amylose is amorphous and is composed mainly of linear chains of α -1,4 linked glucose units with less than 1% α -1,6 branch points (Ball *et al.*, 1998). The degree of polymerisation of amylose is 10^2 - 10^3 . The starch particle also contains proteins, lipids and phosphate, but at a very low concentration (Buleon *et al.*, 1998). The only naturally occurring covalent modification of starch is phosphorylation. About 1/300 glucosyl residues in potato starch are normally phosphorylated on the six-or-three-position; in cereal and leaf starches, the level of phosphorylation is considerably lower than this (Blennow *et al.*, 2000; Smith *et al.*, 2003).

In spite of the simple chemistry of starch, the final structure of starch molecules is variable and complex (Blennow *et al.*, 2002). They are deposited as semicrystalline granules and the dimension varies from 1 μ m-100 μ m diameter in length according to the organ and species in question (Gallant *et al.*, 1997). Starch granules from leaves are usually smaller than granules from storage organs (Martin and Smith, 1995). The basic structure of the granule is dictated by the packing of amylopectine in organized arrays (French, 1984). Amylose molecules appear to exist as single helices within the starch granule, interspersed with amylopectine in amorphous regions (Gidley, 1992; Jane *et al.*, 1992). At the highest level of organization that can be resolved by light microscopy, concentric “growth rings” of alternating crystallinity can be observed that are a few hundred nm thick (Gallant *et al.*, 1997). Each growth ring is composed of 9-nm thick repeating lamellae consisting of 5-7-nm liquid-crystalline lamellae (Waigh

et al., 1998) linked by a 2-4 nm amorphous lamella containing the branch points (Blennow *et al.*, 2002).

In storage organs like tubers, roots or turions, starch serves as a long-term carbon reservoir and its function is connected to the seasonal periods. Usually, a massive degradation of stored starch occurs during germination. In contrast, in photosynthetically competent tissues, starch is transiently accumulated and it is adapted to the daily light/dark cycle and to the circadian rhythm. The function of transitory starch is to provide both reduced carbon and energy during periods unfavourable for photosynthesis.

1.2. Regulation of starch degradation

1.2.1. Enzymes of starch degradation

Degradation of starch in cereal endosperm has been thoroughly investigated over an extended period of time. However, both the pathway and the regulation of starch breakdown are poorly understood in organs other than cereal endosperm. The main reason for this is that the endosperm is acellular at the time of starch degradation. In other type of plant organs, degradation of starch occurs in living cells where the network of regulatory processes is expected to be very complex.

Detailed studies on enzymes which possibly participate in starch degradation such as endo- and exo-amylases (α - and β -amylases, respectively), starch phosphorylase, and the D-enzyme (4- α -glucanotransferase or disproportionating enzyme), have shown that they are present in multiple forms in almost all organs (Trethewey and Smith, 2000). However, starch granules can be directly attacked only by hydrolytic enzymes such as α -amylase (Steup *et al.*, 1983; Witt *et al.*, 1995; Witt and Sauter 1995a; Witt and Sauter 1995b; Witt and Sauter, 1996) and α -glucosidase (Sun and Henson, 1990; Sun *et al.*, 1995). Recently, it was shown that transgenic potato plants, which have a reduced activity of a chloroplast-targeted β -amylase showed a defect in starch degradation (Scheidig *et al.*, 2002). An Arabidopsis knockout mutant with a T-DNA insertion in the D-enzyme gene demonstrated that a D-enzyme is also necessary for normal starch degradation (Critchley *et al.*, 2001). Several starch excess mutants have

been isolated in *Arabidopsis* (Caspar *et al.*, 1989, 1991; Zeeman *et al.*, 1998) which are probably mutated in genes coding for proteins involved in starch degradation. Although many of these enzymes can degrade starch, it is not clear which of these enzymes are involved in starch degradation *in vivo* (Kossmann and Lloyd, 2000). For most of the starch-degrading enzymes, extra-chloroplastidic isoforms contribute greatly to the total activity in leaves (Okita *et al.*, 1979; Steup, 1988; Beck and Ziegler, 1989), hampering the study of those forms that are localized within the chloroplasts. Moreover, the understanding of the starch degradation process is complicated by the fact that starch-degrading enzymes such as starch phosphorylase and the D-enzyme are present in both starch-degrading and starch-synthesizing cells. Most enzymes have no obvious regulatory properties that would prevent their degradation activity during starch synthesis, and it is not clear whether they participate primarily in the synthesis or in the degradation of starch (Takaha *et al.*, 1993; Duwenig *et al.*, 1997; Rentzsch, 1997; Albrecht, 1998;; Takaha *et al.*, 1998). This complex situation indicates that in most cells there are several pathways for starch degradation. The model in Fig.1 represents the possible routes for starch degradation using enzymes known to be present in many plastids.

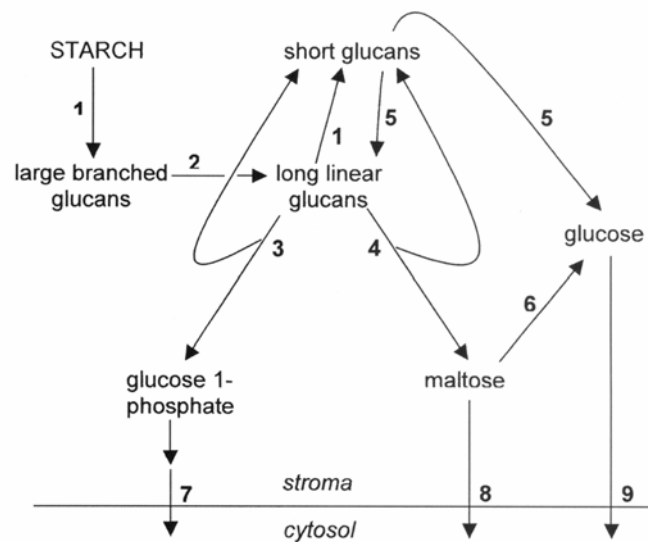


Fig.1: Possible pathways of starch mobilization in a plastid. Reactions catalysed by enzymes known to occur in starch-containing plastids. Note that not all possible reactions are indicated: the actions of β -amylase, starch phosphorylase and disproportionating enzyme are shown only on linear glucans, but these enzymes can also act on the outer chains of branched glucans, provided that these are of sufficient length. In other words, the action of debranching enzyme does not necessarily precede the action of these exo-acting degradative enzymes. 1, α -amylase; 2, debranching enzymes (isoamylase and limit-dextrinase); 3, starch-phosphorylase; 4, β -amylase; 5, disproportionating enzyme; 6, α -glucosidase; 7, triose-phosphate translocator; 8, hypothetical maltose translocator; 9, glucose translocator (Smith *et al.*, 2003).

1.2.2. GWD and its physiological role

The discovery of a new protein, originally named R1, in the matrix of starch granules from potato tubers, represents a breakthrough in the way that enzymatic mechanisms of starch degradation are investigated. This protein with a molecular weight of 160 kDa is associated with the surface of starch granule (Lorberth *et al.*, 1998). Studies on transgenic potato plants have shown that the antisense repression of R1 leads to a strong reduction in the amount of starch-bound phosphate, whereas the expression of R1 in *Escherichia coli* has resulted in an increase in the phosphorylation of bacterial glycogen (Lorberth *et al.*, 1998). In addition, analysis of the starch accumulating *sex 1* (starch excess) mutant of *Arabidopsis* that is defective in the R1 protein have shown a reduction in the phosphate content of leaf starch (below the detection limit in plants lacking R1) (Yu *et al.*, 2001). These data strongly suggest that the R1 protein plays a crucial role in determining the phosphate content of starch. Recently, the biochemical function of R1 protein has been elucidated: R1 catalyzes a dikinase-type of reaction, transferring the γ -phosphate of ATP to water, whereas it transfers the β -phosphate to phosphorylate glucosyl residues of α -glucans both at the C-6 and C-3 positions (Ritte *et al.*, 2002) (Fig.2). Therefore, the appropriate designation of R1 is glucan-water dikinase (GWD, EC. 2.7.9.4).

The ratio of *in vitro* phosphorylation to α -glucans is similar to that occurring naturally in starch (Bay-Smidt *et al.*, 1994; Ritte *et al.*, 2002). Recently, an *in vitro* assay has been described in order to determine the starch-phosphorylating activity in crude plant extracts (Ritte *et al.*, 2003).

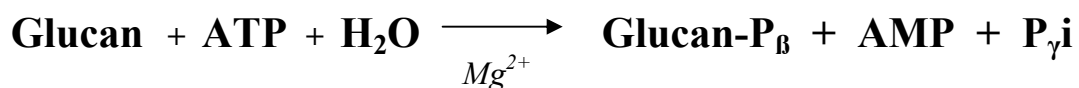


Fig.2: Reaction catalysed by GWD. Phosphorylation occurs in a dikinase-type reaction. GWD phosphorylates itself with β -phosphate and subsequently, transfers the β -phosphate to the glucan indicating a ping-pong reaction mechanism (according to Ritte *et al.*, 2002).

The putative domain structure of the GWD protein includes an N-terminal plastid-targeting transit peptide, two putative starch-binding domains (with homology to an

uncharacterised putative starch-binding domain of α -amylase) and two C-terminal regions representing a phosphohistidine domain and a nucleotide-binding domain (Yu *et al.*, 2001). *Arabidopsis sex1* shows 66.3% identity with GWD from potato, but two other open reading frames with homology to GWD can be identified in the *Arabidopsis* genome (Yu *et al.*, 2001). Both putative translation products in *Arabidopsis* are smaller than GWD and have less similarity (50-30%) to potato GWD. The function of these GWD-s is still unknown, but might be related to the phosphorylation of α -glucans or other metabolites (Blennow *et al.*, 2002).

Using a screening approach, starch granules and soluble proteins from tubers of sweet potato and yam, seeds of maize, barley and banana fruits were recognized by the potato GWD antibodies, suggesting that GWD might be present also in these plants and, therefore, may have a general function in plants (Ritte *et al.*, 2000a). However, some of these plants (maize, pea and barley) synthesize storage starch having a low or undetectable phosphate content. Hence, the assumed presence of GWD does not necessarily result in significant starch phosphorylation.

1.2.3. Starch phosphorylation and starch degradation

The analysis of transgenic potato plants and the *sex1* mutant of *Arabidopsis* have shown strongly impaired degradability of starch. This has led to a repression of cold-sweetening in potato tubers and to starch-excess phenotype in mutant leaves (Lorberth *et al.*, 1998; Yu *et al.*, 2001). In the *sex1* mutant, a general correlation between the reduced level of phosphate in amylopectine and the extent of starch accumulation in leaves has been shown (Yu *et al.*, 2001). These data strongly suggest that starch-phosphorylation is necessary for normal starch degradation to occur.

As a result, the role of GWD (as a starch phosphorylating enzyme) in the starch degradation process represents an important aspect of starch degradation mechanisms. Moreover, the association of GWD to the starch surface depends on the metabolic situation of the cells. Depending on the pre-treatment of *Solanum tuberosum* L. (and from *Pisum sativum* L.) leaves, GWD was bound to the starch granule surface when the leaves were darkened and when they performed net starch degradation. In contrast, following irradiation (during starch synthesis), GWD was recovered

practically in the soluble state (Ritte *et al.*, 2000b). It is assumed that the binding of GWD to the starch granules during net starch degradation is either an important event within the (or essential prerequisite for) starch breakdown process.

However, the precise relationship between GWD, amylopectine phosphorylation and starch degradation is not yet fully understood. As mentioned above, in the *sex1* mutant and in potato antisense plants, the low phosphate content of the leaf starch is related to impaired starch degradation. In contrast, similar phosphate contents in the endosperm starch do not result in starch degradation in cereals (Blennow *et al.*, 2002).

Hence, it is presumed that starch can be degraded using different pathways. In a phosphorylation-dependent pathway, the starch phosphate content provides the signal which could define the initial points of starch degradation recognized by starch degrading enzymes. In organs in which starch synthesis and degradation are tightly regulated, co-localized, and in close temporal connection (as in the transitory starch of leaves), additional regulation caused by starch phosphorylation might be required (Blennow *et al.*, 2002).

1.3. Turions of *Spirodela polyrhiza* as a model system to study degradation of storage starch

Turions are resting fronds of aquatic vascular plants. They are distinguished from normal fronds by their smaller size, lack of parenchyma, high content of storage starch and thicker cell walls (Appenroth and Bergfeld, 1993; Appenroth and Gabrys, 2001). The vegetative fronds cannot tolerate low temperatures and therefore usually die in late autumn (Henssen, 1954). The resting fronds, however, overcome unfavourable seasons by sinking to the bottom of ponds and lakes (Landolt, 1986; Landolt and Kandeler, 1987), playing an important role in the survival strategy of *S. polyrhiza*. Turions also contain two meristematic pockets, from which new vegetative fronds can develop after germination has occurred (Appenroth and Bergfeld, 1993). The newly formed fronds then start a new life cycle.

The main storage compound in turions is starch, which accounts for up to 70% of the total dry mass (Henssen, 1954). As previous studies have shown, storage starch fulfils

two distinct functions (Dölger *et al.*, 1997, Ley *et al.*, 1997). First, starch mobilized over months or years at extremely low rates enables turions to survive during unfavourable germination periods. Secondly, starch supports the growth of newly formed fronds once germination has been initiated. Under the last condition, starch is mobilized at a much higher rate, which leads to its depletion within a few days.

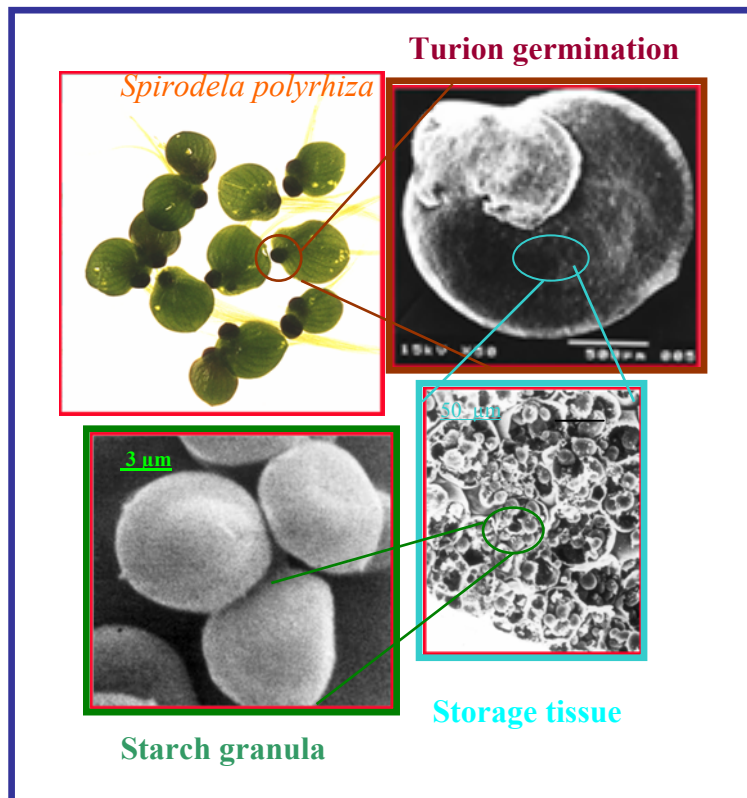


Fig.3: Vegetative sprouts (picture by K-J. Appenroth), and germinating turions of the duckweed *Spirodela polyrhiza* (SEM pictures according to Trilapur *et al.* (1999)) are used as a system to investigate the biochemistry of storage starch degradation.

Germination and degradation of storage starch in turions are induced by light whose effect is mediated by a low fluence response mode of phytochrome (Appenroth and Augsten, 1990; Dölger *et al.*, 1997; Appenroth and Gabrys, 2001). Whereas the germination response can be induced by a single red light (Rp) pulse, the degradation of starch requires irradiation with continuous red light (cR) or the application of repeated, i.e. hourly pulses. This provides the opportunity to initiate starch degradation at any time by using a suitable light treatment.

In other systems like potato tubers (Sowokinos *et al.*, 1985), poplar wood (Witt *et al.*, 1995; Sauter *et al.*, 1998) or Ipomoea batatas (Takahaka *et al.*, 1995), the initiation of starch degradation is more difficult to be induced under experimental conditions. Therefore, the simple way to initiate the degradation of starch in turions as mentioned above represents an advantage of turion system to study the biochemistry of starch breakdown.

Another advantage of the turion system as an experimental model is the separation of degradation and synthesis of starch in such storage organs. In leaves, the processes of synthesis and degradation of transitory starch are co-localized and it is difficult to assess whether or not significant degradation occurs during net starch synthesis (Smith *et al.*, 2003). Because of this heterogeneous background in leaves, it is difficult to study the biochemistry of starch degradation enzymes in this system. In contrast, in *S. polyrhiza* germination and growth of newly-formed sprouts, which is supported by the degradation of storage starch in turions, occur in the spring season (Ley *et al.*, 1997) whereas the formation of turions and that of storage starch proceeds in late summer or autumn (Henssen, 1954).

Thus, the well-characterised inducibility by light and the high rate of starch mobilization make turions of *S. polyrhiza* an excellent system to investigate the biochemistry of storage starch degradation.

1.4. Purpose of the work

The initiation and regulation of starch degradation in storage organs are not yet well understood. We followed the hypothesis that the binding of distinct proteins to the starch particle may be crucial for the process of starch metabolism. In potato leaves, the pattern of proteins bound to starch granules was determined during net starch degradation and during net starch biosynthesis. Unlike most of the compounds, only the protein named R1 (GWD) varied depending upon the pre-treatment of the leaves (dark/light) (Ritte, 1999; Ritte *et al.*, 2000b). The same analysis of potato tubers indicated that there are no differences in the pattern of starch-bound proteins of growing and germinating tubers (Ritte, 1999). However, the detailed characterization of proteins binding to the starch surface during starch degradation is missing.

Functional proteomics of starch-associated proteins provide an improved tool to analyse protein-starch interactions during starch breakdown.

It is assumed that alterations at the starch particle surface are involved in the reversible binding of proteins. Recombinant GWD binds differently to starch granules from dark-kept versus irradiated potato leaves (Ritte *et al.*, 2000b). However, the nature and the regulation of the modifications of the starch surface are unclear. Hence, a precise analysis of the starch surface offers the opportunity to characterize the yet unknown modifications of starch during its breakdown process.

Using the advantages of the turion system, the following questions are to be addressed in this work:

1. Does the phytochrome-mediated light signal, which triggers finally the process of starch degradation, activate GWD?
2. Does the phosphorylation of GWD induce, as a consequence, the phosphorylation of starch?
3. Does the phosphorylation of starch affect the binding properties of starch-associated proteins like GWD and α -amylase?
4. Does the phosphorylation of starch influence the starch degradation *in vitro*?

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

The chemicals were supplied by the following companies: Pharmacia, Uppsala, Sweden; Megazyme Gamma-Chemie, München, Germany; Merck Eurolab, Darmstadt, Germany; Millipore Corporation, Schwalbach, Germany; Roth, Karlsruhe, Germany; Serva Feinbiochemica, Heidelberg, Germany; Sigma-Aldrich Chemie, Deisenhofen, Germany.

The materials and equipment for chromatography (Fractogel EMD TMAE 650, 12ml; and LC organizer and L-6210 pump) were obtained from Merck Eurolab (Darmstadt, Germany), and the radiochemicals were obtained from Hartmann Analytic (Braunschweig, Germany).

2.1.2. Enzymes

- Alkaline Phosphatase(CIAP) (Calf intestine alkaline phosphatase)	MBI Fermentas (St. Leon-Rot, Germany)
- Acid Phosphatase (from potato, grade II)	Roche Diagnostics GmbH (Mannheim, Germany)
- β -amylase (catalogue No. A7005)	Sigma (München, Germany)

2.1.3. Molecularweight standard

Proteins were estimated using the prestained kit MW-SDS-Blue from Sigma-Aldrich Chemie (Deisenhofen, Germany).

2.1.4. Antibodies

The following antibodies were used:

- Polyclonal antisera, raised against α -amylase purified from *Pisum sativum* leaves (Ziegler, 1988) according to Louvard *et al.* (1975), were kindly supplied by Dr. Paul Ziegler, University of Bayreuth, Germany.
- Antibodies raised against the α -glucan-water-dikinase (GWD) (EC 2.7.9.4) holo-protein from *Solanum tuberosum* (Ritte *et al.*, 2000b), were kindly supplied by Prof. Martin Steup (University of Potsdam, Germany).
- Antibodies, raised against β -amylase, were purified from turions of *Spirodela polyrhiza* (Luka *et al.*, 1999).
- Anti-rabbit IgG (A6154; Sigma, München, Germany) covalently bound to Horseradish Peroxidase.

2.1.5. Plant material

All experiments were performed using etiolated turions of the duckweed *Spirodela polyrhiza* (L.) Schleiden, strain SJ (Appenroth *et al.*, 1996).

2.1.6. Light sources

The following light sources and filters were used: red light pulses ($\lambda_{\max} = 683$ nm, half-band width 63 nm, $490 \mu\text{mol m}^{-2} \text{s}^{-1}$, 5 min) were applied using a slide projector (Diafant 250, Liesegang, Düsseldorf, Germany; 24 V/250 W) equipped with glass filter RG645, 3 mm thick (Schott, Mainz, Germany) and a dichroic filter IR7, 3 mm thick (OptoChem, Stromberg, Germany). For irradiation with continuous red light, ($\lambda_{\max}=658$ nm, half-bandwidth 25 nm, $12 \mu\text{mol m}^{-2} \text{s}^{-1}$) red fluorescence tubes, (36 W/60; Osram, München, Germany) plus a red Plexiglas (GS501, 3 mm thick; Röhm, Darmstadt, Germany) were used. All manipulations of the turions were carried out in dim green light ($\lambda_{\max} = 553$ nm, half band width 8 nm, $<0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$) as described

previously (Appenroth *et al.*, 1996). Fluence rates were measured with a radiometer IL 1700 equipped with a detector SED 033 (International Light, Newburyport, USA).

2.2. Methods

2.2.1. Cultivation of turions

Nutrient solution:	60 μ M	KH ₂ PO ₄
	8 mM	KNO ₃
	1 mM	Ca(NO ₃) ₂
	1 mM	MgSO ₄
	25 μ M	Fe(III)EDTA
	5 μ M	H ₃ BO ₃
	13 μ M	MnCl ₂
	0.4 μ M	Na ₂ MoO ₄

S. polyrhiza was vegetatively cultivated under mixotrophic conditions. The cultivation of *S. polyrhiza* and the formation of turions were carried out as described by Appenroth *et al.*, (1996). The nutrient solution was supplemented with 50 mM glucose to enhance the formation of turions (Appenroth *et al.*, 1996). Dormant turions – that do not germinate regardless of the light conditions – were harvested 28 days after the start of the cultivation. To obtain non-dormant turions – that could germinate by phytochrome-mediated signal – cf. Appenroth *et al.* (1999), cold treatment ($5 \pm 1^\circ\text{C}$) was carried out for additional 28 days in continuous darkness. In some series of experiments, phosphate was omitted from the nutrient solution either during cold treatment (5°C for 28 days) or during the subsequent dark/light treatment at 25°C . Finally, orthophosphate was omitted during both the cold period and during the subsequent treatment.

During cold treatment and in all experiments except turion formation, glucose was omitted from the nutrient solution. Except for the cold period ($5 \pm 1^\circ\text{C}$), turions were kept at $25.0 \pm 0.1^\circ\text{C}$.

2.2.2. Irradiation program

Dormant or non-dormant turions were preincubated for three days in darkness at $25.0 \pm 0.1^\circ\text{C}$. Thereafter, they were either kept in darkness (designed as **D**) or irradiated with continuous red light (designed as **cR**) for different periods. In most of the cases, the irradiation was carried out for one day (designed as **1cR**) or two days (designed as **2cR**). When the effect of red light pulses was to be analysed, the cultivated turions were irradiated with red light pulses (2.1.6) followed by a dark treatment for three days (designated as **Rp**). Following various light treatments, turions and newly formed sprouts were immediately frozen in liquid nitrogen and stored at -80°C until use for further analysis.

2.2.3. Isolation of starch

Extraction Buffer (A):	100 mM	HEPES-KOH, pH 8.0
	0.1 mM	EDTA
	5 mM	DTT
	2 mM	benzamidine
	2 mM	aminocaproic acid

Samples (0.25-1.0 g FW) were homogenized in liquid nitrogen using a mortar. The homogenate was vigorously mixed with 4 ml extraction buffer A (see above) (Ritte *et al.*, 2000b), and thereafter, was filtered through two layers of a polyamide net (100 μm and 30 μm mesh width; PA-100/49 and PA-40/30; Franz Eckert, Waldkirch, Germany). The resulting filtrate was centrifuged for 4 min at 1,000 g. The pellet that contains the starch granules was washed with 4 ml buffer A and then resuspended in 2 ml of the same buffer. For the purification of the starch granules, this suspension was layered on the top of a 5 ml cushion consisting of 95% (v/v) Percoll (Amersham-Pharmacia, Freiburg, Germany) which was dialysed for 15 h against 50 vol of distilled water and 5% (v/v) 0.5 M HEPES-KOH, pH 7.0 (pH of the solution was around 7.5) and was centrifuged for 15 min at 2,000 g. This purification step was repeated twice. The resulting starch fraction was then washed twice with buffer A (5 ml and 1 ml, respectively), frozen in liquid nitrogen and dried under vacuum for 8 h. The samples were stored at -80° until use.

2.2.4. Starch quantification

Starch was quantified according to Ley *et al.* (1997).

2.2.5. Analyses of starch-related proteins

2.2.5.1. Extraction of soluble proteins

Samples (0.25-1.0 g fresh weight (FW)) were homogenized in liquid nitrogen using a mortar. The homogenate was vigorously mixed with 4 ml extraction buffer A (2.2.3) and 125 mg of insoluble polyvinylpyrrolidone (Polyclar AT; Serva, Heidelberg, Germany). The homogenate was centrifuged for 20 min at 10,000 g. The resulting supernatant was collected and freshly used for soluble protein analyses.

2.2.5.2. Extraction of starch-associated and starch-internalized proteins

To analyse starch-associated and starch-internalized protein fractions, no Polyclar AT was added in the homogenate (2.2.2 and 2.2.5.1). Starch-associated proteins were released from granules by incubating 5 - 25 mg purified starch granules (2.2.2) in SDS-containing sample buffer (Laemmli 1970). Usually, a volume of 20 µl of the buffer was used per mg starch dry weight (DW). The suspension was incubated for 10 min at room temperature under continuous rotation using the mixing rotor „Variospeed“ (Renner, Darmstadt, Germany). Subsequently, the suspension was centrifuged for 5 min at 16,000 g and the supernatant (containing the starch-associated proteins, see Morrison and Karkalas, 1990; Mu-Forster *et al.*, 1996) was collected. When starch-associated proteins were to be analysed by 2D-E, the extraction of starch-associated proteins was performed using 0.125 M Tris-HCl, pH 6.8 and 2% SDS as described above. To extract proteins trapped inside the granules (starch-internalized proteins), the remaining starch pellets were washed twice for 5 min in SDS-containing sample buffer (30 µl per mg starch (DW)) and the supernatants were discarded. The granules were resuspended in an equal volume of the same buffer and were then boiled for 5 min. Finally, samples were centrifuged as above and the supernatants were collected.

2.2.5.3. Protein determination

The soluble proteins were quantified by Bradford (1976) and Appenroth *et al.* (1982) using BSA as a standard. The starch-associated proteins were quantified by the bicinchoninic acid method (Sigma, Taufkirchen, Germany) according to the manufacturer's instruction.

2.2.5.4. Protein Gel-Electrophoresis

2.2.5.4.1. SDS-polyacrilamide gel electrophoresis (SDS-PAGE)

One dimensional polyacrilamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed as described by Laemmli (1970). Soluble proteins (2.2.5.1) (2 vol) were mixed with (1 vol) 3x concentrated SDS-sample buffer and denaturated by boiling for 3 min. When the soluble proteins were analysed, equal amounts corresponding to the total amount of protein (2.2.5.3) were loaded onto gel. After the extraction, the fractions of starch-associated and starch-internalized proteins (2.2.5.2) were denaturated by boiling for 3 min. Equivalent amounts of protein, corresponding to the same amount of starch (usually 5 mg starch (DW)), were loaded onto gel.

2.2.5.4.2. Two dimensional SDS-PAGE (2D-E)

The extracted starch-associated proteins (2.2.5.2), or the soluble proteins (2.2.5.1), were precipitated by the methanol/chloroform method as described by Hippler *et al.* (2001). The procedure for 2D-E was also carried out as described by Hippler *et al.* (2001). Each sample contained protein corresponding to the same amount of starch (25 mg starch DW, 350 µg total protein) when starch-associated proteins were analysed, or 200 µg of the total protein when soluble proteins were analysed. Proteins were dissolved in 380 µl of solubilisation buffer (2 M thiourea, 8 M urea, 4% (w/v) 3-[[Cholamidopropyl]dimethylammonio]-1propane-sulfonate (CHAPS)], 20 mM Tris, 30 mM DTTe, 0.5% (v/v) immobilized pH gradient (IPG) buffer, pH 3-10 (Amersham Bioscience Europe GmbH, Freiburg, Germany), 0.05% β-dodecyl-maltoside and 0.5% (v/v) bromphenol blue) and incubated for 1 h at room temperature, then centrifuged at

9000 x g for 5 min. From the supernatant, 350 µl were carefully removed, filled into a fresh tube and 0.5% (v/v) IPG buffer (Amersham Bioscience Europe GmbH, Freiburg, Germany) was added again. This solution was loaded into the groove of a ceramic strip tray (Amersham Bioscience Europe GmbH, Freiburg, Germany). The IPG strip, with a linear pH gradient from 3.0 to 10.0, was overlayed upside down, covered with paraffin and incubated for 12 h at 20°C. After rehydratisation of the IPG strips in the presence of the sample, electrophoresis was performed using an IPGphor apparatus (Amersham Bioscience Europe GmbH, Freiburg, Germany) at 15°C until 60 kVh were reached.

Solution 1:	50 mM	Tris-HCl, pH 6.8
	6 M	Urea
	30% (v/v)	Glycerol
	2% (w/v)	SDS
	2% (w/v)	DTT

Solution 2:	50 mM	Tris-HCl, pH 6.8
	6 M	Urea
	30% (v/v)	Glycerol
	2% (w/v)	SDS
	2.5% (w/v)	Iodoacetamide
	0.5% (w/v)	Bromphenol blue

After electrophoresis in the first dimension was finished, the IPG strips were removed from the tray and equilibrated for SDS-PAGE using two different equilibration solutions. The IPG strips were first incubated with Solution 1 (see above) for 12 min and, afterwards, with Solution 2 (see above) for 5 min. The strips were then briefly washed with water, loaded on the top of a prepared SDS-PAGE (8% acrylamide; 0.42% piperazine diacrylamide (PDA)) and covered with 0.5% agarose. The SDS-PAGE was run at 8°C and 30 mA per gel using a BIO-RAD multi-cell apparatus.

2.2.5.5. Protein staining on gel

Proteins were detected in gels by silver staining (Blum *et al.*, 1987).

2.2.5.6. Electrophoretic transfer

Transfer buffer:	192 mM	Glycin
	25 mM	Tris
	10 % (v/v)	Methanol

To transfer proteins from the SDS-PAGE gel onto a Hybond-P (PVDF) membrane (Amersham Bioscience Europe GmbH, Freiburg, Germany), the semidry technique was applied using the HEP1 blot apparatus from PEQLAB (Owl Separation System, Peqlab Biotechnology GmbH, Erlangen, Germany). The transfer was performed for 1-2 h at constant 400 mA.

2.2.5.7. Immunostaining

PBS buffer:	75.0 mM	NaCl
	3.0 mM	KCl
	1.5 mM	KH ₂ PO ₄
	4.5 mM	NaHPO ₄ , pH 7.5

B-PBS-T buffer:	1x	PBS
	5% (w/v)	Skimmed milk powder
	1% (v/v)	Tween 20

PBS-T buffer:	1x	PBS
	0.1% (v/v)	Tween 20

After the transfer, the membranes were incubated for 1 h in B-PBS-T buffer and then they were incubated for 2 h with the primary antibodies (2.1.4) which were diluted in B-PBS-T as follows:

1: 5 000	α -amylase
1: 7 000	GWD
1: 2 500	β -amylase

After a washing procedure in B-PBS-T, the membranes were incubated for 1 h with secondary antibodies (2.1.4) in B-PBS-T buffer (dilution: 1:10 000) and subsequently, washed in PBS-T buffer. Peroxidase activity was detected according to the supplier's instructions (ECL; Amersham-Pharmacia, Freiburg, Germany) using a high performance chemiluminescence film (hyperfilm ECL, Amersham, Life science, UK). Immunosignals were quantified using an ImageMaster VDS system (Amersham-Pharmacia, Freiburg, Germany) and the software supplied by the producer. For quantification, aliquots of a serially diluted extracts were applied to the SDS-PAGE gel and the intensities of the immunosignals were monitored.

2.2.6. Protein purification

Grinding buffer (B):	100 mM	citric acid-NaOH, pH 6.0
	5 mM	Ca-acetate

Grinding buffer (C):	100 mM	HEPES-KOH, pH 8.0
	1 mM	EDTA
	2 mM	benzamidine
	2 mM	aminicaproic acid

FPLC buffer:	50 mM	HEPES-NaOH (KOH for GWD), pH 8.0
	1 mM	EDTA
	5 mM	DTT

α -amylase was partially purified from turions of *S. polyrhiza* in order to:

- 1) confirm that the polyclonal antibodies raised against α -amylase from *P.sativum* (Ziegler 1988) recognized this enzyme in the extracts of *S. polyrhiza*.
- 2) for use as a probe in binding studies *in vitro*.

Turions (0.5 g FW) were homogenized in 8 ml of a grinding buffer (B) and the homogenate was centrifuged for 20 min at 39,000 g. Following centrifugation, the supernatant was passed through a membrane (GVWP, 0.22 μm pore size; Millipore, Schwalbach, Germany) and was then applied to an anion exchange column (fractogel EMD TMAE 650, 12 ml, Merck, Darmstadt, Germany) using the FPLC buffer (see above) and a linear sodium chloride gradient (0-200 mM). The flow rate was 1 ml min^{-1} . Fractions (2 ml each) that contained α -amylase activity were pooled and the proteins were precipitated by adding solid ammonium sulphate (to 60 % saturation), pelleted by centrifugation and dissolved in 0.5 ml buffer A (2.2.3) without benzamidine and aminocaproic acid. The proteins were fractionated using a Superdex 200 prep grade column (Pharmacia, Uppsala, Sweden; 9 X 30 mm; flow rate of 3.6 ml h^{-1}) equilibrated with buffer A (see above). Fractions of 0.6 ml were collected. Aliquots of the activity containing fractions were subjected to SDS-PAGE and Western analysis. The specific activity of α -amylase in the crude extract was 3.0 μmol^{-1} (g protein $^{-1}$) and was increased to 245 μmol^{-1} (g protein $^{-1}$) resulting in 82-fold purification.

GWD protein was partially purified from turions of *S. polyrhiza*. 1g (FW) turions were homogenized in 10 ml of the grinding buffer (C) in the presence of 500 mg Polyclar AT and the homogenate was centrifuged for 20 min at 39,000 g. Subsequently, the supernatant was passed through a membrane (GVWP, 0.22 micrometer pore size; Millipore, Schwalbach, Germany) and was applied to the anion exchange column (Fractogel EMD TMAE 650) using the fast performance liquid chromatography system (FPLC) as described above. The FPLC buffer (for GWD, see above) contained a linear sodium chloride gradient (0-200 mM). The flow rate was 1 ml min^{-1} . Fractions (2 ml each) were collected and 100 μl from 40 each fraction were mixed with 500 μl of 6x concentrated Laemmli buffer, denaturated and separated by SDS-PAGE. With GWD antibodies, Western analyses were performed and the fractions with the highest intensity of immuno signal of GWD were determined. Five fractions from the collected ones that contained the highest amount of GWD were pooled. The proteins were fractionated using the Superdex 200 prep grade column (see above) equilibrated with grinding buffer (C) without benzamidine and aminocaproic acid. Fractions of 0.6 ml were collected and the three resulted fractions

with the highest amount of GWD (Western analysis with GWD antibodies as above) were combined and used for *in vitro* binding studies.

β -amylase was partially purified from turions of *S. polyrhiza* to investigate the binding at the surface of starch granules *in vitro*. Soluble proteins were extracted from turions (1 g FW) as described above. Following centrifugation, the supernatant was subjected to anion exchange chromatography (fractogel EMD TMAE 650) as described for the purification of α -amylase (see above). Fractions (1 ml each) that contained β -amylase activity were pooled and the proteins were precipitated by adding solid ammonium sulphate (to 60 % saturation), pelleted by centrifugation and dissolved in 0.5 ml buffer A (2.2.3) without benzamidine and aminocaproic acid. Subsequently, proteins were fractionated on a Superdex 200 column as used for the purification of α -amylase (see above). Those three eluted fractions (1 ml each) that contained the highest β -amylase activities were combined and used for binding studies *in vitro*. The specific activity of β -amylase in the crude extract was $15.7 \mu\text{mol s}^{-1}(\text{g protein})^{-1}$ and was then increased to $1\,080 \mu\text{mol s}^{-1}(\text{g protein})^{-1}$, resulting in a 72-fold purification. In the final β -amylase preparation, α -amylase activity was undetectable.

2.2.7. Enzyme activity assays

To measure α -amylase activity, the so-called „ $\beta\beta\beta$ -method“ (Ziegler, 1990) was employed using β -limit dextrin as a substrate (Megazyme; supplied by Gamma-Chemie, München, Germany). The assay contained:

280 μl	0.5 M acetic acid-NaOH, pH 6.0
20 μl	50 mM CaCl_2
200 μl	0.5 % (w/v) cyclodextrine
50 μl	β -amylase solution (23 units)
200 μl	plant extract

Total: 750 μl

This mixture was incubated for 60 min at 30°C to degrade endogenous soluble starch. Subsequently, 250 μl of β -limit dextrin (1 % w/v) were added. At intervals

(immediately following the addition of β -limit dextrin, 15 min and 30 min later) aliquots (200 μ l each) were withdrawn and treated with the dinitrosalicylic acid reagent as described by Sharma and Schopfer (1982). Activity is expressed in most cases as nmol maltose formed per g DW and s. The activity of β -amylase was determined as described by Luka *et al.* (1999).

2.2.8. *In vitro* binding of starch-related proteins to starch granules

2.2.8.1. De-proteinisation of starch granules

Proteins can be removed from the starch granules surface using either toluene (Morrison and Karkalas, 1990) or SDS (Ritte, 1999).

Toluene treatment

Starch granules (5 mg DW) were vigorously mixed for 30 seconds at room temperature with 1 ml of toluene-water saturated mixture (3:1) and separated by centrifugation (5 min; 16,000 g). This procedure was repeated four times. Subsequently, the starch granules were washed three times with 1 ml of water and separated by centrifugation as before.

SDS treatment

Starch granules (5 mg DW) were incubated for approximately 50 min at room temperature with 1 ml 0.5% SDS and separated by centrifugation as above. The granules were washed for 30 min with water, subsequently incubated for around 30 min in 100 mM HEPES-NaOH, pH 8.0; 1 mM EDTA, and finally separated by centrifugation as above.

2.2.8.2. *In vitro* binding of proteins to starch

Starch granules (5 mg (DW)) were deproteinised using Toluene (2.2.8.1.). The deproteinised starch granules were incubated a) with the coarse plant extract from turions of *S. polyrhiza* (500 μ l containing 610 μ g total protein) (see 2.2.5.1), or b) with the partially purified α -amylase (500 μ l containing 200 μ g of the total protein) (see 2.2.6); partially purified GWK (500 μ l containing 130 μ g of the total protein)

(see 2.2.6). The suspensions were incubated with the starch granules for 1 h at 4°C using the mixing rotor „Variospeed“ (15 rpm). Controls were incubated in the respective buffers without protein. After incubation, the suspension was placed on the top of 5 ml cushion consisting of 95% (v/v) Percoll and 5% 0.5 M HEPES-KOH, pH 7.0 and centrifuged for 15 min at 2,000 g . Subsequently, the starch granules were washed twice in 1 ml of 100 mM HEPES-KOH, pH 8.0 and the starch-associated proteins were extracted (2.2.5.2) and investigated by SDS-PAGE and Western analysis.

When the *in vitro* binding of β -amylase was to be analysed, the following modifications were done:

1. For the turion homogenization, the volume of buffer A (2.2.3) was increased from 4 ml to 40 ml.
2. Native starch granules were not de-proteinised.

The *in vitro* binding experiment with β -amylase was carried out as described above. Partially purified β -amylase (2.2.6) in buffer A (500 μ l containing 200 total μ g protein) was used.

2.2.9. *In vitro* degradation of starch granules

2.2.9.1. Standard procedure

Starch granules (10 mg (DW)), isolated from turions irradiated as stated, were incubated in 0.5 ml starch degradation buffer at 25°C under continuous rotation using the mixing rotor „Variospeed“ (15 rpm).

Starch degradation buffer:	50 mM	HEPES-KOH, pH 7.5
	1 mM	EDTA

In another experiment, starch granules isolated from turions irradiated for 24 h were firstly deproteinised using Toluene (2.2.8.1) and subsequently, incubated in starch degrading buffer. Also, 20 mg (DW) commercial potato starch (EC No 232-686-4

Sigma-Aldrich) were incubated in starch degrading buffer as described above. The two last samples were used as controls in the case of incubation in the absence of native starch-associated proteins. Following incubation, the starch granules were separated by centrifugation (5 min; 16,000 g) and the supernatant was used to measure the amount of sugars released from the starch granules during the incubation time. A modified method as described by Waffenschmidt and Jaenicke (1987) was applied. 0.4 ml of the supernatant were hydrolysed for 100 min at 100°C after adding 0.4 ml HCl 1 M. After a short centrifugation (5 min; 16,000 g), 0.5 ml of the supernatant was neutralised by adding 0.25 ml of 1M NaOH. For the colorimetric determination (absorbance 560 nm) of the released D-glucose, 0.2 ml of the neutralised solution were brought to 1 ml volume adding 0.3 ml water and 0.5 ml working reagents, as described by Waffenschmidt and Jaenicke (1987). The amount of glucose released is given in microgram glucose/g starch granules (DW).

2.2.9.2. *In vitro* degradation of de-proteinised starch granules after *in vitro* binding of soluble proteins

Starch granules (10 mg DW) isolated from turions kept in darkness or irradiated for 24 h were deproteinised with toluene (2.2.8.1). Soluble proteins from 24 h irradiated turions were extracted (2.2.5.1) and a volume of 500 µl containing 570 µg total protein was incubated with the starch granules under continuous rotation (15 rpm), for 1 h at 4°C. Controls were incubated in buffer without soluble proteins. Following incubation, the suspension was placed on the top of 5 ml cushion consisting of 95% (v/v) Percoll and 5% 0.5 M HEPES-KOH, pH 7.0 and centrifuged for 15 min at 2,000 g. Subsequently, the starch granules were washed twice with 1 ml of 100 mM HEPES-KOH, pH 8.0 and after a final centrifugation step (5 min; 16,000 g) the *in vitro* degradation assay (standard procedure) was performed (2.2.9.1).

2.2.10. Phosphatase treatments

2.2.10.1 Acid phosphatase treatment and analysis of GWD

Starch granules (30 mg DW) were incubated in 1 ml buffer containing 100 mM PIPES-HCl, pH 6.0; 1 mM DTT in the presence of 10U potato acid phosphatase

(Roche Diagnostics GmbH, Mannheim, Germany). The following controls were used: 1) buffer without starch, and 2) buffer without acid phosphatase. The suspension was slowly rotated (10 rpm) for 20 min at 26°C, using the mixing rotor „Variospeed“. After incubation, the suspension was centrifuged for 5 min at 16,000 g. The supernatant, which contains the starch-associated proteins released from starch granules during the phosphatase treatment, was precipitated with Phenol-Ether (Sauve *et al.*, 1995) and the pellet was resolved in 0.1 ml SDS buffer (Laemmli, 1970). This fraction was termed „released proteins“. The starch pellets separated by centrifugation were used for the extraction of starch-associated proteins (2.2.5.2) which remained bound on the starch granules after phosphatase treatment. The level of GWD in the „released proteins“ fraction (see above) was analysed by SDS-PAGE/ Western blotting and was compared with the starch-associated fraction. The changes of the pattern of starch-associated GWD were investigated by 2D-E/ Western analysis (2.2.5.4.2).

2.2.10.2. Acid phosphatase treatment and *in vitro* starch degradation

In vitro degradation of starch granules under the effect of acid phosphatase was performed following the phosphatase treatment described above (2.2.10.1). The controls were incubated without phosphatase. After the incubation with acid phosphatase, starch granules were separated by centrifugation (5 min; 16,000 g), and were shortly washed twice with 1 ml of 50 mM HEPES-KOH, pH 7.5. Subsequently, the assay of starch degradation *in vitro* was performed as described (standard procedure: 2.2.9.1).

2.2.10.3. Alkaline phosphatase (CIAP) treatment and analysis of GWD

Starch granules (20 mg DW) were incubated in 0.5 ml buffer containing 50 mM Tris-HCl, pH 7.5 and 1 mM MgCl₂ for 10 min at 30°C and then 20U CIAP were added. In the controls, starch granules were incubated in buffer without CIAP. The suspension was slowly rotated for 15 min at 30°C, using the mixing rotor „Variospeed“ (10 rpm). The reaction was stopped by adding 10 mM Na₃PO₄ (Ahmand and Huang, 1981). The suspension was centrifuged for 5 min at 16,000 g. The supernatant (which contains the starch-associated proteins released from starch granules during the phosphatase

treatment) was mixed 1 : 1 with 2x sample buffer (Laemmli, 1970) and denaturated for 3 min at 95°C. The starch pellets separated by centrifugation were used for the extraction of starch-associated proteins (2.2.5.2) that remained bound on the starch granules after phosphatase treatment. The level of GWD in the „released proteins“ fraction (see above), in comparison to the starch-associated fraction, was investigated by SDS-PAGE and Western analysis.

2.2.10.4 Alkaline phosphatase treatment and *in vitro* starch degradation.

In vitro degradation of starch granules under the influence of alkaline phosphatase was performed after the phosphatase treatment described above (2.2.10.3). The controls were incubated without phosphatase. After the incubation with alkaline phosphatase, starch granules were separated by centrifugation (5 min; 16,000 g) and thereafter, were shortly washed twice in 1 ml of 50 mM HEPES-KOH, pH 7.5. Subsequently, the assay of starch degradation *in vitro* was performed as described (2.2.9.1).

2.2.11. Treatment with non-radioactive and radioactive-labelled ATP

2.2.11.1. Non-radioactive ATP assay and analysis of GWD

ATP assay buffer:	50 mM	HEPES-KOH, pH 7.5
	1 mM	EDTA
	6 mM	MgCl ₂
	5 µl	Protease inhibitor cocktail for plant cell and tissue extract (P9599; Sigma-Aldrich)
	0.1 mM	ATP (in 10 mM Tris-HCl, pH 7.5)

Starch granules (30 mg (DW)) were incubated in 0.5 ml ATP assay buffer. Controls were incubated without ATP. The suspension was rotated for 20 min at 25°C, using the mixing rotor „Variospeed“ (15 rpm). Subsequently, the suspension was centrifuged for 5 min at 16,000 g, and the starch pellets were used for the extraction of associated proteins (2.2.5.2). The changes of the pattern of starch-associated GWD were investigated by 2D-E/ Western analysis (2.2.5.4.2).

When soluble GWD was to be analysed, 1g (FW) from 24 h irradiated turions was used. In this case, for the extraction of soluble proteins, buffer A (2.2.3) was modified using 50 mM HEPES-KOH, pH 7.5. The concentration of total soluble protein was 100 µg in 0.5 ml. The incubation conditions (with or without ATP in the controls) were the same as for the starch granules described above. After incubation, the sample with soluble proteins was used to investigate changes of the GWD pattern by 2D-E/ Western analysis.

The non-radioactive ATP assay was carried out with recombinant GWD, kindly offered by Prof. M. Steup, University of Potsdam; see Ritte *et al.* (2002). In this case 0.3 µg recombinat GWD were used for the non-radioactive assay as described above, followed by 2D-E/ Western blotting.

2.2.11.2. Non-radioactive ATP assay and starch degradation *in vitro*

When the effect of ATP on starch degradation *in vitro* was analysed, the non-radioactive ATP assay was applied (2.2.11.1), with the following modifications:

1. 10 mg starch were suspended in 0.5 ml ATP assay buffer (2.2.11.1). The controls were incubated in the same amount of buffer without ATP.
2. The incubation time was increased to 2 h.

The degradation products were analysed according to the standard procedure as described (2.2.9.1). In another set of experiments, the effect of recombinant GWD on the degradability of starch granules was analysed. Starch granules (10 mg DW) were deproteinised with toluene (2.2.8.1) and then incubated in 0.5 ml buffer ATP-assay buffer (2.2.11.1) with 0.24 µg recombinat GWD. The controls were incubated a) in ATP-assay buffer (2.2.11.1) without recombinat GWD and b) in buffer (2.2.11.1) without ATP. The incubation time was increased to 1 h. Finally, the degradation products were analysed according to the standard procedure as described (2.2.9.1).

2.2.11.3. Radioactive ATP assay and analysis of GWD

The following experiments were carried out by Dr. K.-J. Appenroth and Mrs. Gabriele Lenk. For the incubation of starch granules with radioactive ATP, two different labelled ATP preparations were used: γ -[^{33}P]-ATP (Hartmann Analytic, Braunschweig, Germany) and a mixture of β -[^{33}P]-ATP and γ -[^{33}P]-ATP (designated respectively as γ -ATP and $\beta\gamma$ -ATP) obtained by enzymatic randomization with myokinase and pyruvate kinase (Ritte *et al.*, 2002). Starch granules (30 mg DW) were incubated at 25°C for 30 min in 400 μl of the following solution:

50 mM	HEPES-KOH, pH 7.5
1 mM	EDTA
10 % (v/v)	Glycerol
5 mM	MgCl ₂
5 mM	KCl
100 μM	ATP (containing 4MBq $\beta\gamma$ -ATP; or γ -ATP)

Following incubation, starch-associated proteins were separated by 2D-E and electrophoretic transferred on PVDF membranes. These membranes were used in a phosphoimager (Storm 820, Molecular Dynamics, Krefeld, Germany) to detect radioactive labelled proteins and for immunostaining using GWD antibodies.

2.2.11.4. Calculation of isoelectric points of GWD following potential phosphorylations

The isoelectric points of GWD subjected to multiple potential phosphorylations were calculated using the algorithm from ExPASy's Compute pI/Mw program provided under scansite.mit.edu (Bjellqvist *et al.*, 1993). Two GWD proteins were used for the calculations: R1 from *Solanum tuberosum* (CAD 88947) and sex1 from *Arabidopsis thaliana* (NP 563877).

2.2.11.5 Radioactive ATP treatment and analysis of starch granules

The following experiments were carried out by Dr. K.-J. Appenroth and Mrs. Gabriele Lenk. $\beta\gamma$ -ATP was formed as described above. 10 mg starch (DW) were incubated in 0.5 ml of the following buffer:

50 mM	HEPES-KOH, pH 7.5
1 mM	EDTA
6 mM	MgCl ₂
25 μ M	ATP containing 0.4 MBq $\beta\gamma$ -ATP

After 20 min and 120 min incubation under rotation (15 rpm) at 25°C, the reaction was stopped by adding 120 μ l of 0.1M HCl. The starch granules were thoroughly washed (once with 2% (w/v) SDS and six times with 2mM phosphate-buffered ATP) as described by Ritte *et al.* (2002). Following centrifugation (5 min, 16,000 g), the starch granules were suspended in 0.8 ml of water and mixed with 1.2 ml of scintillation solution (Rothiszint mini; Roth, Karlsruhe, Germany). Radioactivity was measured in a scintillation counter (LS6500; Beckmann, München, Germany). Data were given as differences between the results measured after 20 min and after 120 min incubation. As a control, γ -ATP was used for incubation.

2.2.12. Scanning electron microscopy (SEM)

The investigations by SEM were carried out with the help of Dr. W. Fischer (Institut of General Botany and Plant Physiology, University of Jena, Germany). Freshly isolated starch granules were dehydrated in graded acetone series, with a final passage in 100% acetone and critical-point-dried. Specimens were sputter-coated with gold and observed with JEOL JSM-880 scanning electron microscope at 15 kV (cf. Trilapur *et al.*, 1999).

2.2.13. Non-contact atomic force microscopy (nc-AFM)

High-resolution, nc-AFM work was carried out with the help of Dr. J. Szymonska at the Department of Chemistry, Agricultural University, Krakow, Poland. This nc-AFM

was performed using a Park Scientific Instrument Autoprobe CP model (California, USA) as described by (Krok *et al.*, 2000 and Szymonska *et al.*, 2000). Starch granules isolated from darkened or 24 h irradiated turions were spread onto an adhesive tape fixed onto an AFM sample holder, and observed at ambient conditions. The granules were partially embedded in the „sticky tape” in order to overcome problems with the large height variation in granule topography. For each starch sample, images of six starch granules were collected.

2.2.14. High Performance Anion Exchange Chromatography-Amperometric Detection (HPAEC-PAD) analysis

HPAEC-PAD was performed in order to analyse the nature of the products released from starch degradation *in vitro*. The degradation assay was performed *in vitro* as described above (2.2.9.1.). In the controls, a deproteinisation step using Toluene (2.2.8.1.) was carried out before running the *in vitro* degradation assay. After the separation by centrifugation (5 min 16,000 g) of starch granules from the products released during the *in vitro* degradation, aliquots (400 µl) of the supernatant were passed through a Microcon YM-10 column (Millipore, Schwalbach, Germany) which was equilibrated before with 1 ml of 10 mM HEPES-KOH, pH 7.5. Subsequently, the aliquots were dried under vacuum and used for HPAEC-PAD analysis. This work was carried out with the help of Dr. Nora Eckermann (University of Potsdam).

3. RESULTS

3.1. Protein-starch interactions during starch degradation in turions

3.1.1. Pattern of starch-related proteins analysed by SDS-PAGE and 2D-E

To analyse the pattern of starch related proteins, prior to and during starch degradation, non-dormant turions of *S. polyrhiza* were either kept in darkness (D), or were irradiated for 48 h with continuous red light (2cR). The proteins of the two starch preparations were analysed by SDS-PAGE followed by silver staining (Fig.4). A two-step solubilisation procedure was performed and therefore, two protein fractions were obtained from each starch granule preparation: proteins bound to the starch granule surface (designed as starch-associated proteins), and those trapped inside the starch matrix (designated as starch-internalized proteins). Only one major starch-internalized protein (having a molecular weight of approximately 65 kDa) was detected in starch samples from both darkened and irradiated turions (Fig.4, lanes 2 and 4). In contrast, the pattern of starch-associated proteins was complex and varied depending on the metabolic state of turions (Fig.4, lanes 1 and 3).

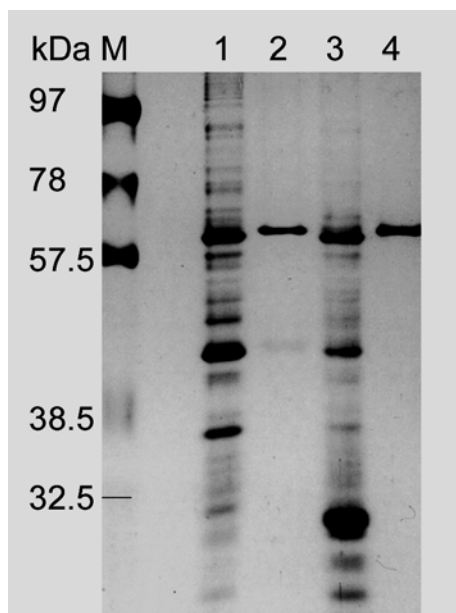


Fig.4: SDS-PAGE of starch related proteins from turions of *S. polyrhiza* as visualized by silver staining. Non dormant turions were either darkened (lanes 1, 2) or irradiated for 48 h with cR (lane 3, 4). Proteins were extracted by incubation with SDS-containing buffer at room temperature (lanes 1, 3: starch-associated proteins) or by boiling the SDS-washed granules (lanes 2, 4: starch-internalized proteins).

Using a proteomic approach as a tool to investigate in more details the interaction between proteins and starch in turions, starch-associated-proteins extracted from turions kept in darkness (D) or irradiated for 24 h (1cR) and 48 h (2cR) were analysed by separation at high

resolution (isoelectric focusing/SDS-PAGE) two dimensional gel electrophoresis followed by silver staining (Fig.5). These analyses showed that more than 100 spots representing starch-associated proteins were detectable in each treatment. Comparing 2D-E protein maps, clear differences between the darkened and irradiated turions were observed. These differences can be summarized as follows: a) selective starch-protein binding was affected by irradiation, since protein spots appeared/disappeared in the irradiated samples, (see Fig.5, red and blue arrows respectively); b) several proteins might have undergone the process of post-translational modifications during irradiation (see Fig.5, green arrows).

3.1.2. Native and artificial binding of starch-related proteins

The complexity of pattern of starch-associated proteins (Fig.4 and Fig.5) might be overestimated because several soluble proteins can artificially bind to the granule surface during tissue homogenization. This possibility was tested by isolating the starch granules a) according to the standard procedure (2.2.3) and b) using a volume of buffer A 10 and 100-fold greater than in the standard procedure. Under the last conditions (b), artificial protein-starch binding was expected to be diminished. In both cases, the starch-associated proteins were extracted and separated by SDS-PAGE. Blots of the starch-associated proteins were probed using antibodies directed against the α -glucan-water-dikinase (GWD) from potato, α -amylase from pea and β -amylase from turions of *S. polyrhiza*. Proteins of about 160 kDa, 40 kDa, and 60 kDa were recognized by these antibodies which suggests that GWD, α -amylase and β -amylase are associated with starch in turions (Fig.6a). When the isolation of starch from turions irradiated with a single red pulse (Rp) and kept for three days in darkness, was carried out with 100-fold buffer volume, neither the level of GWD, nor that of α -amylase decreased significantly (Fig.6a; 6b). On the contrary, only traces of β -amylase (approximately 5% of the quantity obtained by the standard procedure) could be detected when 100-fold buffer volume was used (Fig.6a; 6b). Similar results were obtained upon testing two isoforms of α -glucosidase from turions: α -glucosidase I (optimum pH for the enzyme assay pH 5.7, molecular weight 58 kDa) and α -glucosidase III (optimum pH for the enzyme assay pH 7.1, molecular weight 71 kDa; Appenroth, personal communication).

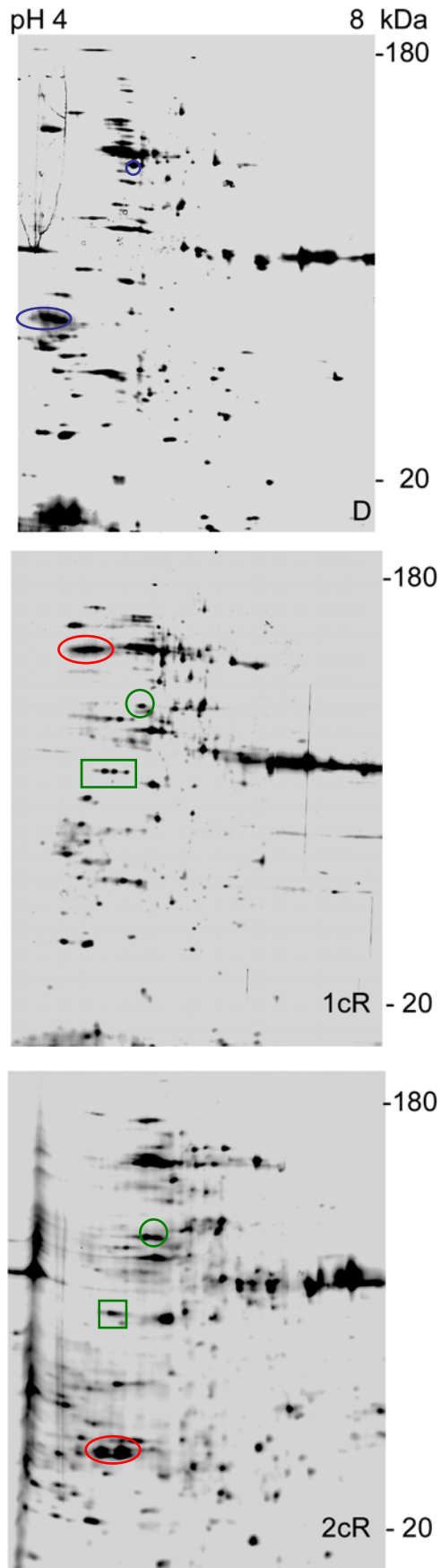


Fig.5: Starch-associated proteins extracted from turions kept in darkness (D) or irradiated for 24 h (1cR) and 48 h (2cR) were separated by 2D-E and visualized by silver staining. The red circles show the presence of new proteins spots compared to (D); the blue ones represent proteins spots which disappear during irradiation, and the respective green circles and squares represent possible post-translational modifications of proteins. Representative pictures of at least three parallel experiments of each treatment are shown.

Both isoforms were clearly detectable by Western blot analysis when the starch granules had been isolated using the standard procedure (2.2.3), but the signal intensity was decreased to approximately 8% (isoform I) or was below the limit of detection (isoform III) when the 100-fold buffer volume was used (Fig.6a). These results suggest that all three proteins, α -glucosidase I, α -glucosidase III and β -amylase, most likely become attached to the starch granules during the grinding procedure whereas the starch-association of GWD and α -amylase appears to be physiologically relevant. Therefore, all further studies on *in vivo* protein-starch binding were focused on the two latter proteins, GWD and α -amylase.

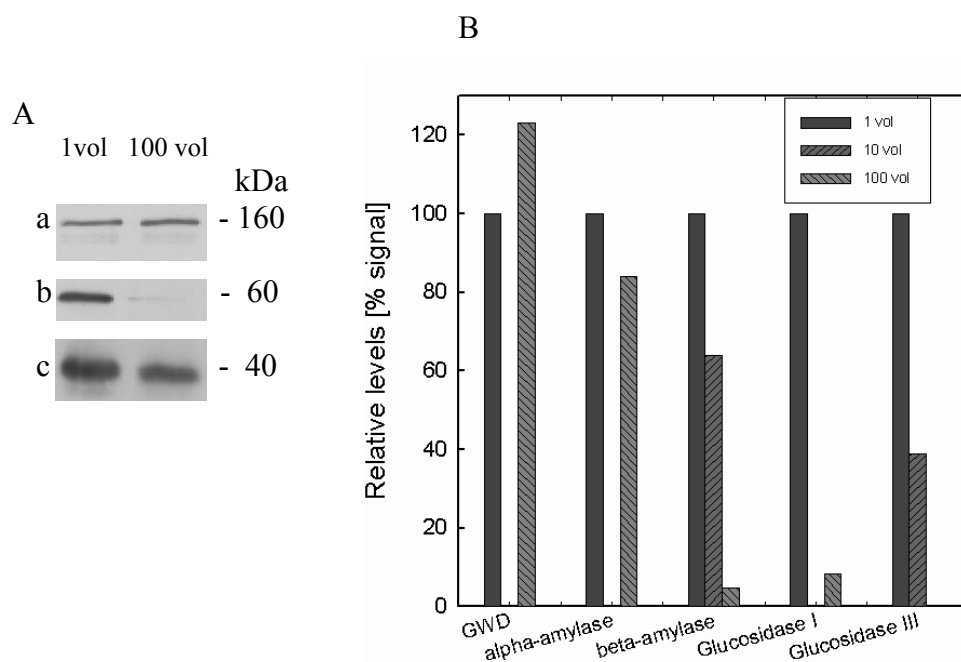


Fig.6: Standard and modified procedure for the preparation of starch-associated proteins from turions of *S. polyrhiza*. Turions were irradiated with a short red pulse and kept for 3 days in darkness (Rp). Starch-associated proteins were isolated according to the standard procedure (dark columns = 100%). Alternatively, a 10 or 100-fold volume buffer was used for the homogenization (hatched columns). Subsequently, the extraction of starch-associated proteins was carried out identically and proteins were subjected to SDS-PAGE and Western analysis. Data are given as a percentage of the controls (isolated according to the standard procedure with 1 volume buffer). A: Western blot of a) GWD; b) β -amylase; c) α -amylase. B: The relative levels of Western signals including those of glucosidases I and III.

3.1.3. Analysis of GWD and α -amylase

3.1.3.1 Starch-associated and soluble pools of the GWD and of α -amylase

Germination and starch degradation of turions were induced by irradiation with cR for 3 days and the newly formed fronds were mechanically separated from turions. Subsequently, three protein fractions (soluble proteins, starch-associated proteins and starch-internalised proteins) were obtained from both turions and fronds. Each fraction was analysed by SDS-PAGE, and the relative levels of GWD and α -amylase were determined immunochemically. By far the largest proportion of the two proteins was recovered in the soluble fraction. Approximately 70% of the total GWD was found in the soluble fraction of fronds, whereas more than 90% of the total α -amylase was detected in the soluble fraction of turions (Table 1). Only 1% of the GWD and 4% of the α -amylase were starch-associated in the starch preparation obtained from turions, and their content in starch-internalised fraction was negligible.

Fraction	GWD		α -amylase	
	<i>Turions</i>	<i>Sprouts</i>	<i>Turions</i>	<i>Sprouts</i>
<i>Soluble</i>	27	72	91	5
<i>Starch-associated</i>	1	ND	4	ND
<i>Starch-internalized</i>	0.07	ND	ND	ND

Table 1: *Compartmentation of GWD and α -amylase in turions and newly formed fronds of S. polyrhiza.* Non-dormant turions were irradiated for 3 days in cR. The newly formed fronds were separated from the turions. Soluble, starch-associated and starch-internalised proteins were extracted, separated by SDS-PAGE and analysed by Western blotting. For each protein, data is given as a percentage of the sum of the 3 fractions. The data was taken from a single experiment. The values obtained in another experiment deviated less than 7% from those presented here. ND = below the detection limit of the method applied.

3.1.3.2. Soluble pools of GWD and α -amylase in turions and the effect of light

To analyse the initial period of starch degradation, the soluble fractions of GWD and of α -amylase were determined in non-dormant darkened or irradiated turions. The turions were first subjected to cold treatment and transferred to 25°C (time 0; Fig.7). After 3 days of darkness, the turions were irradiated (either with cR or with a 5 min Rp followed by darkness), or were kept in continuous darkness (control). Following the homogenization of turions and centrifugation, the soluble GWD and α -amylase were quantified

immunochemically in the supernatant. In addition, the activity of the soluble α -amylase was measured. The soluble pool of GWD increased slightly in darkness (Fig.7a). Four days after a single Rp, the level of soluble GWD was slightly higher than in continuous darkness. Under either of these conditions, net starch degradation was not yet initiated (Dölger *et al.*, 1997). The increase of soluble GWD was much stronger during cR. This light treatment induced starch degradation (Fig.7a). In contrast, neither the total protein level nor the activity of α -amylase changed significantly after any of the light applications (Fig.7b; 7c).

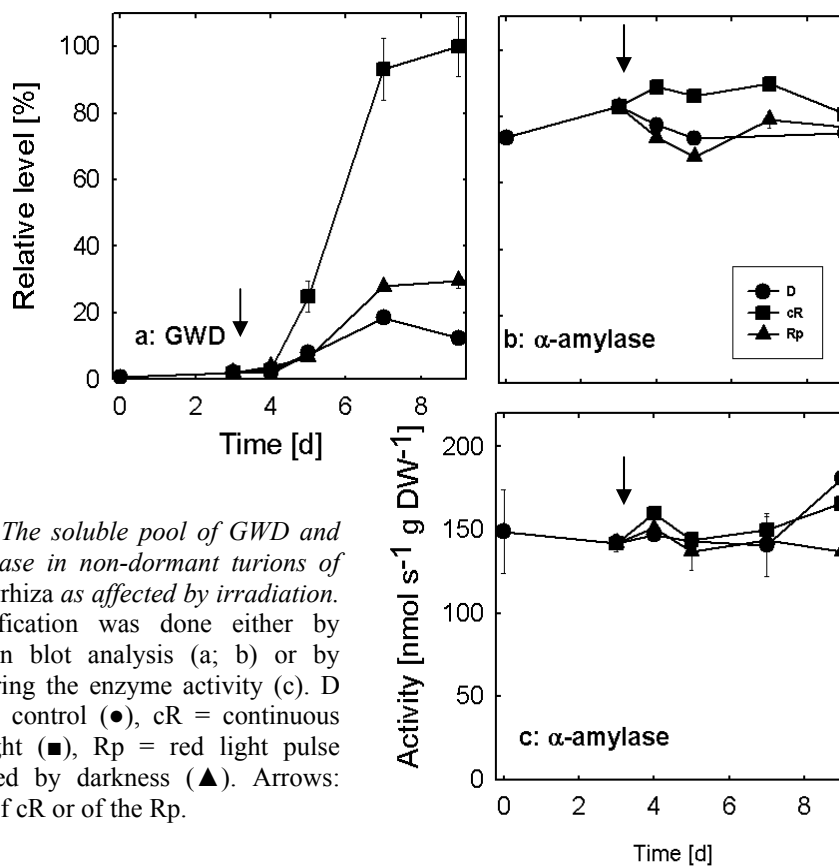


Fig.7: The soluble pool of GWD and α -amylase in non-dormant turions of *S. polyrhiza* as affected by irradiation. Quantification was done either by Western blot analysis (a; b) or by measuring the enzyme activity (c). D = dark control (●), cR = continuous red light (■), Rp = red light pulse followed by darkness (▲). Arrows: onset of cR or of the Rp.

3.1.3.3. Starch-associated GWD and α -amylase under different starch degrading conditions

Both dormant and non-dormant turions were used to study the relative amounts of starch-associated GWD and of α -amylase. In addition, the starch content of the turions was monitored. In agreement with a previous report (Ley *et al.*, 1997), the starch content of dormant turions remained constant, irrespectively of dark or light conditions (Fig.8A: left panels). In non-dormant turions, however, a massive starch degradation strictly depended on continuous irradiation (Fig.8A: right panels).

To investigate a possible correlation between starch degradation and the level of starch-associated proteins, turions were either kept in continuous darkness or were irradiated with cR for six days. In all samples, proteins were extracted from equal amounts of starch suspended in equal volumes of buffer. In non-dormant turions, the amount of the starch-associated GWD, as monitored by Western blot analysis, remained essentially constant during the first 36 hours of cR and then declined (Fig.8A: upper right panel). The amount of starch-associated α -amylase increased within the first 24 hours of irradiation and subsequently decreased to below the detection limit (Fig.8A: upper right panel). The original Western blots are presented in Fig.8B.

In non-dormant turions irradiated with cR, two effects are evident: firstly, a remarkable transient increase of the level of α -amylase and, secondly, a drop in the level of both GWD and α -amylase that precedes the decrease of the starch content (Fig.8A: upper right panel). The relevance of these two effects for starch degradation is stressed by the fact that they were observed neither in darkness (Fig.8A: lower right panel) nor in dormant turions regardless of the light conditions (Fig.8A: left panels). In dormant turions, the starch-associated level of both GWD and α -amylase increased in essentially the same manner during the first 3 or 4 days in both darkness and continuous light (Fig.8A: left panels).

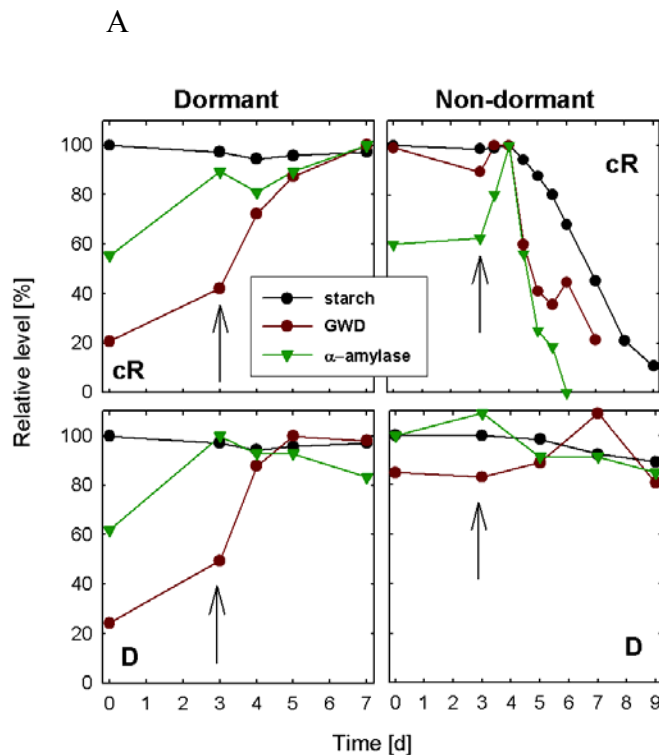
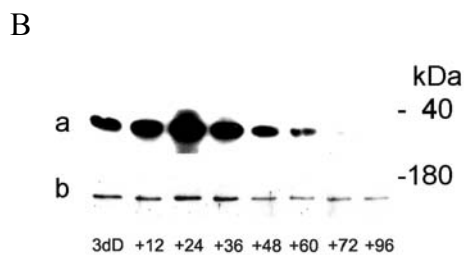


Fig.8: Effect of light treatment on the level of starch-associated proteins and starch contents in dormant and non-dormant turions of *S. polyrhiza*. A: Dormant and non-dormant turions. Turions were either irradiated with cR for the time given (cR) or were kept in continuous darkness (control; D). Dormant (left panels) and non-dormant (right panels) turions were used. (●) = starch content; (●) = level of GWD; (▼) = level of α -amylase. The set of data is based on four independent experiments. Standard errors of the means (3-9%) were omitted for clarity. Arrows: Start of the light treatment.



B: Western blots of GWD and α -amylase in non-dormant turions. Turions were irradiated with cR for the time indicated. Starch-associated proteins were isolated, separated by SDS-PAGE and detected by Western analysis. Numbers indicate hours of light treatment (cR) of the turions following a dark treatment for 3 days (3dD).

3.1.3.4. Influence of external phosphate on starch degradation and on the level of GWD and α -amylase

Taking into consideration that GWD catalyses the phosphorylation *in vitro* of several starch-like glucans (Ritte *et al.*, 2002) and thereby may affect the starch degradability, it was investigated how the supply of phosphate affects the correlation between starch degradation and starch-associated levels of GWD and α -amylase *in vivo*. In the following set of experiments, the influence of the exogenous phosphate on the degradability of starch granules in turions was tested. Three sets of experiments were designed: phosphate was omitted from the nutrient solution a) either during the cold treatment (5°C for 28 days) that is required to break dormancy or b) during the subsequent light/dark treatment (3 days darkness plus 4 days cR) at 25°C or c) during both the cold period and subsequent treatment. Table 2 shows that maximal starch degradation was obtained when phosphate was present both during cold period and during the subsequent treatment at 25°C. When phosphate was omitted during either of these two periods, less starch was degraded. However, when both treatments were performed in the absence of exogenous phosphate, almost no starch degradation was measured (Table 2). Thus, the rate of the light-dependent net starch degradation can be modulated by external phosphate. The correlation between the phosphate-modulated starch degradation and the levels of starch-associated fraction of GWD and that of α -amylase is shown in Fig.9. Phosphate deficiency resulted in a lower rate of starch degradation (Table 2, Fig.9). Under these conditions, elevated levels of starch-associated GWD and α -amylase were observed (Fig.9).

Cold-treatment medium	Light/dark treatment medium	Starch-content [g g ⁻¹ DW]
+P	+P	Dark control: 0.8 ± 0.01
+P	+P	0.2 ± 0.01
+P	-P	0.4 ± 0.01
-P	+P	0.3 ± 0.01
-P	-P	0.7 ± 0.02

Table 2: Effect of external phosphate supply on the starch content in turions of *S. polyrhiza*. Cold treatment (5°C) was carried out in the presence (60 µM; +P) or absence (-P) of phosphate in otherwise complete nutrient solution. The subsequent treatment (25°C; 3 days of darkness plus 4 days cR) was carried out either in the presence (60µM; +P) or in the absence (-P) of orthophosphate. Dark control: no light treatment.

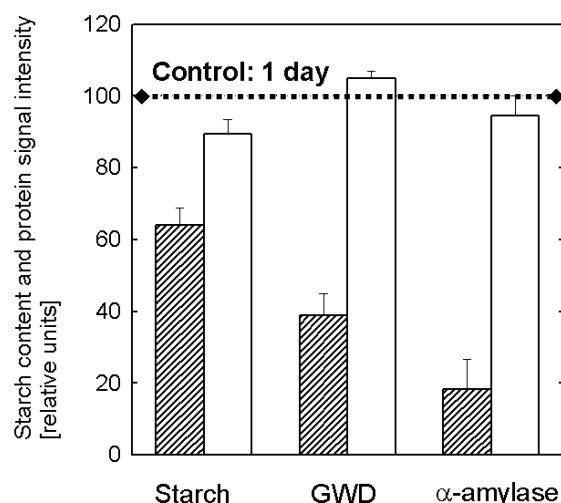


Fig.9: Effect of external phosphate on starch content and on the level of starch-associated proteins, GWD and α-amylase in non-dormant turions of *S. polyrhiza*. Turions were cold-treated in a phosphate-deficient medium. Following the transfer to 25°C, phosphate was supplied (+P; 60 µM). Alternatively, no phosphate was added (-P). The turions were darkened for additional 3 days and were then irradiated for 3 days with cR. The starch contents were determined. Starch-associated GWD and α-amylase were quantified by Western blot analysis. Data is given as a percentage of the samples that had been kept in darkness for 3 days (= D control, 100% as marked by dotted line).

3.1.3.5. Influence of maltose, maltodextrine, detergents (Triton X-100 and SDS) and toluene on the starch-binding capacity of proteins

The protein-starch binding capacity was tested by incubating native starch granules with maltooligosaccharides and Triton X-100 *in vitro*. Native starch granules were isolated from turions kept for 3 days in darkness. Subsequently, the starch granules were washed in extraction buffer A (2.2.3) without test substances. The starch-associated proteins were extracted, separated by SDS-PAGE and the level of bound GWD and α-amylase was determined by Western analysis (Fig.10). The starch-binding capacity of GWD and α-amylase was not affected by the *in vitro* incubation with maltose (Fig.10A). On the contrary, after the incubation with maltodextrine (a mixture of maltooligosaccharides with a polymerisation

grade between G2-G6), about 50% of the GWD and α -amylase were removed from the starch granules (Fig.10B). GWD was also removed from the starch surface after the *in vitro* incubation with Triton X-100 (Fig.10C). Using a serial of concentrations from 0.1 to 1%, it was possible to remove GWD almost totally from the starch surface at a concentration of 0.5% (v/v). In the case of α -amylase, even the concentration of 1% was not affecting the protein-starch binding strength (Fig.10C). Treatment with SDS and Toluene (as described in 2.2.8.1) was efficient in deproteinizing the starch granules (data not shown) used for *in vitro* binding studies as follows.

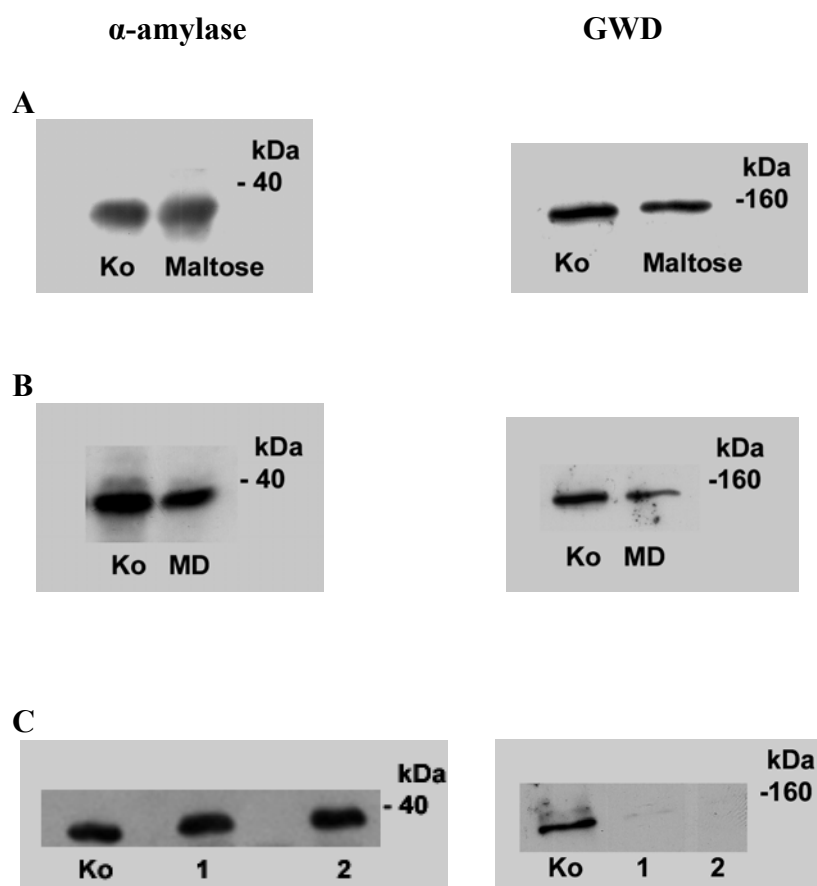


Fig.10: Treatment of starch granules with maltooligosaccharides and detergents *in vitro*. Starch granules were incubated for 30 min at 4°C with 0.5 ml extraction buffer in the presence of (A): 50 mM maltose, (B): 30 mg/ml Maltodextrine and (C): 0,5% (line 1) and 1% (line 2) Triton X-100. Subsequently, granules were centrifuged and the pellets were washed in buffer without Malto-oligosaccharides or Triton. The controls were incubated only in extraction buffer (designated in this Fig. „Ko“). The associated proteins were extracted, separated by SDS-PAGE and investigated by Western blots using antibodies against α -amylase (left side) and GWD (right side).

3.2. Characterisation of starch-associated proteins by 2D-E

3.2.1. Post-translational modifications of starch-associated GWD

3.2.1.1. Pattern of GWD during starch degradation

Starch-associated proteins from turions kept in darkness or irradiated for 24 h and 48 h were extracted. For further characterisation, the starch-associated proteins were separated by 2D-E and investigated by subsequent Western analysis (Fig.11). The GWD antibodies recognized one spot of an abundant protein (isoelectric point (IP) around 5.5; 154 kDa) in the case of darkened (Dark) and 24 h irradiated turions (1cR) (Fig.11). In addition, at least two more spots with the same molecular mass, but different IPs (around 4 and 5 see arrows; 154 kDa) were detected after 24 h irradiation (Fig.11/1cR). When the plant samples were irradiated for 48 h (2cR), the last spots were the only ones recognized by GWD antibodies whereas the abundant protein spot seen before was absent (Fig.11/2cR). These findings demonstrated that the pattern of GWD polypeptides was modified during irradiation.

The soluble pool of GWD was also characterised by 2D-E and Western analysis (Fig.11/sol). The GWD antibodies recognized only the abundant protein spot (iso-electric point around 5.5; 154 kDa) and the protein pattern was not modified in respect to irradiation (data not shown).

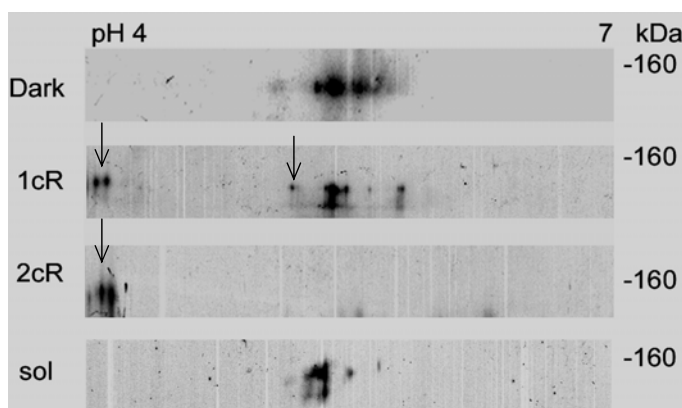


Fig.11: Western blots of starch associated GWD after separation by 2D-E. Gels were focused between pH 3 and 10 and the region of 160 kDa after immunostaining is shown. Except the last line (sol = soluble fraction), starch-associated fractions were investigated. Arrows indicate the position of spots which appeared after irradiation of turions. Dark = dark-kept turions; 1cR = 24 h irradiation; 2cR = 48 h irradiation. sol: Western blots analysis of soluble GWD (1cR) after separation by 2D-E. Arrows: shift on the protein spots.

When recombinant GWD from potato was analysed by 2D-E and Western blotting, a pattern very similar to that of soluble GWD was obtained (see Fig.13/-ATP).

3.2.1.2. Changes on GWD pattern by *in vitro* treatment with ATP

To test the hypothesis that the polypeptide pattern of GWD modified by light was caused by phosphorylation, starch granules isolated from turions kept in darkness (D), or irradiated for 24 h (1cR), were treated with ATP. Subsequently, the starch-associated proteins were extracted and separated by 2D-E. Modifications on the pattern of GWD were investigated by Western analysis (Fig.12). Clear differences between the ATP treated starch granules (+ATP) and the controls (starch granules not treated with ATP;(-ATP)) were observed for both darkened and irradiated turions. The GWD antibodies recognized additional spots with the same molecular mass, but different IPs (shifted into a more acidic region) in the blot from dark samples treated with ATP (Fig.12/D+ATP). On the contrary, in the case of samples irradiated for 24 h, no additional spots were recognized by GWD antibodies when ATP treated samples were investigated. However, the protein spot at a lower IP showed higher immune signal intensity after the ATP treatment (Fig.12/1cR+ATP). The last finding suggested that the new spots observed in light treated samples were caused by phosphorylation of GWD.

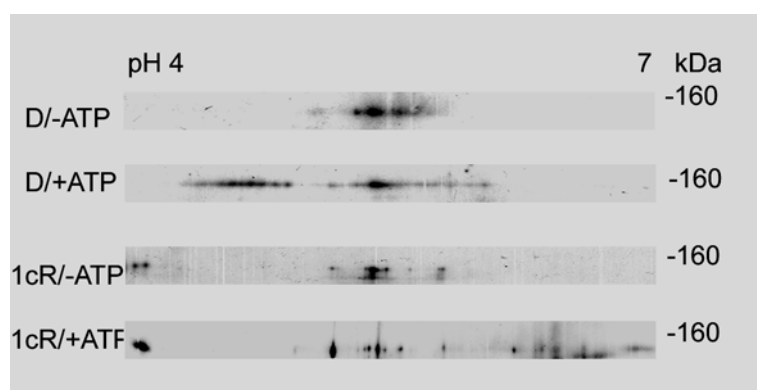


Fig.12: Western blots of starch-associated GWD after the treatment with ATP. Turions were irradiated with red light for 1 day (1cR) or were dark-adapted (D). Starch granules were isolated, incubated with ATP (+ATP) or without ATP (-ATP), starch-associated proteins were extracted, and investigated by 2D-E/Western analysis.

The non-radioactive ATP treatment was also carried out to test possible modifications in the pattern of soluble fraction of GWD isolated from turions and recombinant GWD from potato. In these experiments soluble proteins extracted from turions irradiated for 24 h (1cR) and 0.3 µg recombinant GWD were treated with ATP, separated by 2D-E and probed with GWD antibody after Western transfer (Fig.13). The pattern of soluble GWD from *S. polyrhiza* did not change in spite of ATP treatment (Fig.13 sol/-ATP; Fig.13 sol/+ATP). When recombinant GWD was treated with ATP, GWD antibodies recognized additional spots with the same molecular mass, but with lower IPs (isoelectric point around 4) (Fig.13 rec/-ATP; Fig.13 rec/+ATP). However, these spots showed very low intensity and were barely detectable. It

was evident that the pattern of *in vivo* starch-associated GWD after 24 h irradiation and that of recombinant GWD after ATP treatment were qualitatively similar (Fig.11 and Fig.13).

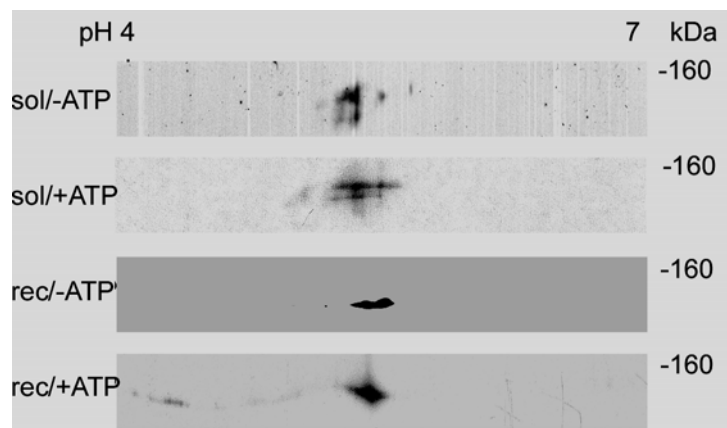


Fig.13: Western blots of soluble GWD after treatment with ATP. Soluble GWD (sol) was extracted from turions irradiated for 24 h in cR. Alternatively, recombinant purified GWD (rec) was used. The protein solutions were incubated with (+ATP) or without (-ATP) and investigated by 2D-E/Western analysis.

To confirm that the light-induced spots in the acidic region represent phosphorylated forms of GWD, starch granules were incubated with radioactive ATP. The granules were isolated from irradiated turions (1 day cR) and either common γ -ATP or randomised $\beta\gamma$ -ATP were used for incubation. Subsequently, the starch-associated proteins were extracted, separated by 2D-E and transferred to a PVDF membrane. The same membranes were used for immuno detection of GWD using antibodies, and for phosphoimaging (Fig.14). The intensity of the immuno signals in the acidic range (Fig.14B) clearly showed successful phosphorylation of GWD by the ATP treatment (cf Fig.12). Almost identical immunoblots were obtained after incubation with $\beta\gamma$ -ATP. No radioactive signal of any polypeptide in the investigated range of molecular weights was detected when γ -ATP was used (Fig.14A). However, using randomised $\beta\gamma$ -ATP those spots detected by immuno staining in the acidic region were also detected to be labelled by radioactivity (Fig.14C). This shows that phosphate in the β -position was used specifically to phosphorylate the protein recognized by GWD antibodies.

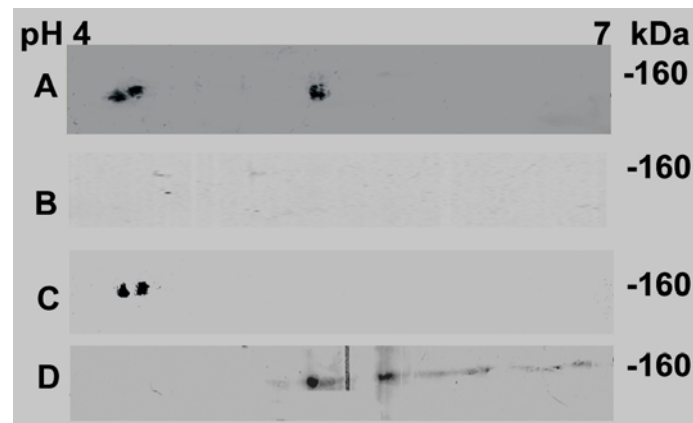


Fig.14: Comparison of Western blots analysed by immuno staining with GWD antibodies, and by phosphoimaging. A: Starch granules were isolated from irradiated turions (1cR) and incubated with [γ - 33 P]ATP. Starch associated proteins were extracted thereafter and analysed after 2D-E and Western transfer by immuno detection using antibodies raised against GWD. Almost identical results were obtained after incubation with [β γ - 33 P]ATP instead of [γ - 33 P]ATP (data not shown). B: The same membranes as in A but were analysed by phosphoimaging to detect radioactive labelled proteins. Starch granules were isolated from irradiated turions (1cR) and incubated with [γ - 33 P]ATP. C: The experiment was carried out as described in A but randomized [β γ - 33 P]ATP was used for incubation followed by phosphoimaging. D: Starch granules were isolated from irradiated turions (1cR) and treated with acid phosphatase before investigated by 2D-E/ Western analysis using antibodies raised against GWD (see below).

3.2.1.3. Modifications of the pattern of starch associated GWD by treatment with acid phosphatase

The mobility of dephosphorylated proteins during isoelectric focusing is expected to be different from phosphorylated proteins. To further characterise the light-dependent phosphorylation of GWD, an acid phosphatase from potato was used and possible modifications of the GWD pattern after the phosphatase treatment were investigated. Starch granules irradiated for 24 h were isolated and incubated *in vitro* with acid phosphatase. Subsequently, the starch-associated proteins were extracted and the pattern of GWD was investigated by Western blot analysis of 2D-E separated proteins. The pattern of GWD was modified after the phosphatase treatment (Fig.14D). The GWD antibodies recognized only the abundant protein spot, whereas the lower IPs spots were absent. The phosphatase treated samples showed a similar Western signal pattern as dark control samples (compare Fig.11/Dark with Fig.14D).

3.2.1.4. The calculated shift of the isoelectric point of GWD by potential phosphorylations

The shift in the position of the 2D-E spots recognized by GWD antibodies from dark-adapted samples and from cR-induced samples was approximately 1.5 pH units. The computer

program provided at scansite.mit.edu (see Experimental Procedures for details) has been used to check this change of the isoelectric point assuming multiple phosphorylation. Because the sequence of GWD from *S. polyrhiza* was not known, the analogous proteins from potato and *Arabidopsis* were used. The isoelectric points of the two proteins in the non-phosphorylated form were calculated to be 5.96 and 5.66, respectively, in accordance with the measured value of approximately 5.5 for GWD from *S. polyrhiza* (Fig.11). In both cases, the calculated shift of the isoelectric point caused by one phosphorylation site is approximately 0.04 pH units. Even ten phosphorylation events would shift the isoelectric point by not more than 0.33 units.

3.2.2. α -amylase is not post-translationally modified during *in vivo* starch degradation

To test the possible reason for the accumulation of α -amylase on the starch granules during 24 h irradiation, two kinds of experiments were carried out:

- 1) Characterization of the polypeptide pattern of α -amylase using 2D-E technique
- 2) *In vitro* protein-starch binding assays using isolated soluble α -amylase and native starch granules from turions.

3.2.2.1. Pattern of α -amylase during *in vitro* starch degradation

Starch granules from turions kept in darkness (D) or irradiated for 24 h (1cR), were isolated. Starch associated proteins were extracted, separated by 2D-E and investigated by Western analysis. α -amylase antibodies recognized one single protein spot (IP approximately 8.3, molecular weight 38 kDa). No differences were observed when darkened and 24 h irradiated α -amylases were compared (Fig.15/D and Fig.15/1cR). It can be concluded that during 24 h irradiation the pattern of α -amylase was not modified.

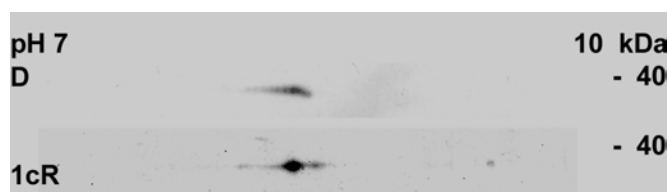


Fig.15: Western blots of starch-associated α -amylase after the separation by 2D-E. Gels were focused between pH 3 and pH 10 and the region of 40 kDa after immunostaining is shown. (D) = dark-kept turions and (1cR) = turions irradiated for 24 h.

3.2.2.2. *In vitro* binding of the soluble α -amylase on starch

Starch granules from turions irradiated for 24 h (1cR) were isolated and the native associated proteins were removed using the toluene de-proteinisation procedure. Soluble proteins were extracted from turions kept in darkness (D) or 24 h irradiated (1cR). The amount of soluble α -amylase was not changed by irradiation (see Fig.7b). Equal amounts of soluble proteins were used for incubation of the starch granules. Subsequently, the starch bound proteins were extracted. The amounts of soluble α -amylase extracted from turions kept in darkness, or irradiated for 24 h that were bound on the starch granules after incubation, were determined by Western analysis (Fig.16/A). Controls were incubated without protein. The amount of α -amylase binding on the starch granules by *in vitro* incubation was not affected by the irradiation of the plant samples used as a source of the soluble protein. The intensity of the immuno signals, which correspond to the protein-starch binding levels, were the same, in spite of irradiation Fig.16/(D+) and Fig.16/(1cR+). This finding confirms that the *in vivo* accumulation of α -amylase on starch granules after 24 h irradiation is not caused by modifications of the α -amylase protein itself.

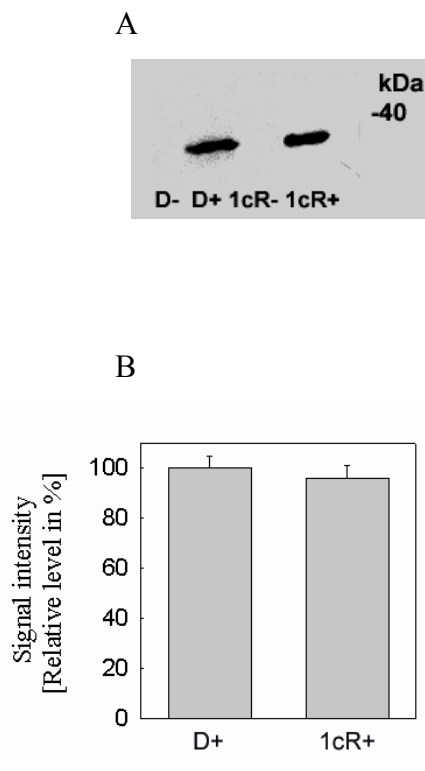


Fig.16: *In vitro* binding of α -amylase on starch. A: Starch granules isolated from turions irradiated for 24 h were de-proteinised. Soluble proteins were extracted from turions kept in darkness (D) or 24 h irradiated (1cR). Equal amounts of soluble protein were incubated with de-proteinised starch granules (D+) and (1cR+). Controls were incubated in buffer without soluble proteins (D-) and (1cR-). The bound proteins were extracted, separated by SDS-PAGE and investigated by Western analysis using α -amylase antibodies. B: Relative intensities (in %) of the Western signals represented in A.

3.3. Modification of starch surface during starch degradation

Possible modifications of starch surface were investigated during starch degradation in turions using the following approaches:

- 1) *In vitro* binding of starch-related proteins to the surface of the starch granules.
- 2) Scanning electron microscopy.
- 3) Non-contact atomic force microscopy (nc-AFM).

3.3.1. *In vitro* binding of GWD, α -amylase and β -amylase

Turions irradiated for 96 h (4cR) were used to extract GWD. This protein was partially purified and used as a probe to investigate possible changes of *in vitro* binding to the surface of starch caused by irradiation. Starch granules were isolated from turions kept in darkness or irradiated for 96 h. The granules were de-proteinised using toluene and subsequently, incubated with a concentrated fraction of GWD containing 130 μ g (in 500 μ l) total protein. As a control, de-proteinised starch granules were incubated in buffer without protein. After incubation and washing the proteins bound to the starch surface were extracted and separated by SDS-PAGE. GWD antibodies were used for Western analysis (Fig.17/upper part). As shown in Fig.17, the capacity of the granule surface to bind GWD depended on the dark/light pre-treatment of turions used for starch isolation: this level was higher when starch granules were isolated from dark-kept turions than when isolated from irradiated turions.

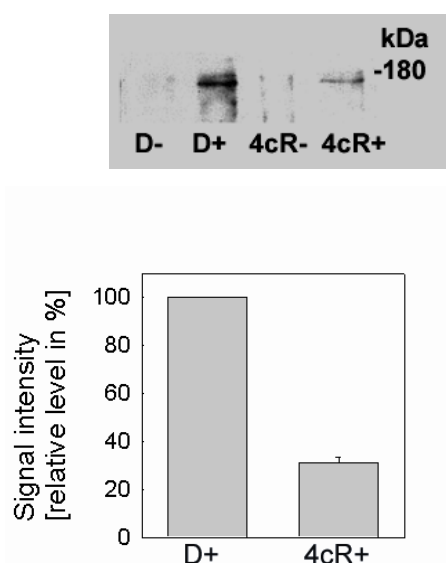
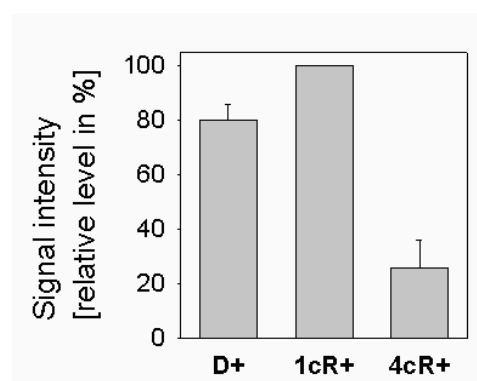
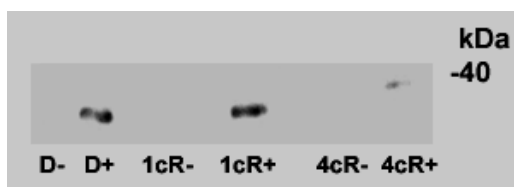


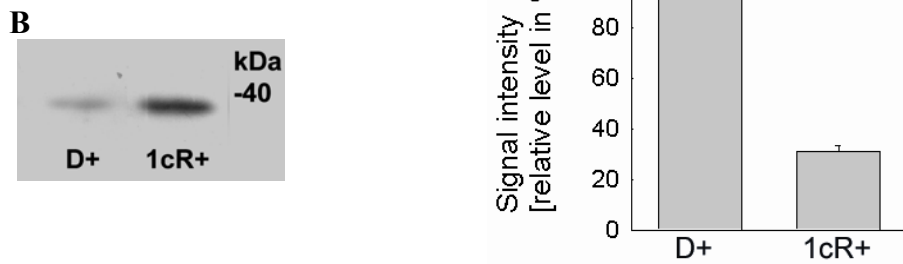
Fig.17: *In vitro* binding of GWD to starch granules. Upper part: starch granules were isolated from turions kept in darkness (D) or irradiated for 96h (4cR), then de-proteinised and, subsequently, incubated with partially purified GWD extracted from turions irradiated for 96 h (indicated with +). Controls were incubated in buffer without GWD (D-) and (4cR-). Lower part: Relative intensities (in %) of the respective Western signals.

To test further the influence of dark/light pretreatment of turions on possible changes of starch surfaces, two other proteins were also used as probes: a) α -amylase and b) β -amylase from turions.

a) Starch granules were isolated from turions kept in darkness (D), or irradiated for 24 h (1cR) and 96 h (4cR). The starch-associated proteins were removed from the starch surface using toluene. Soluble proteins were extracted from turions irradiated for 24 h. 1) The coarse extract (610 μg total protein in 500 μl), and 2) partially purified α -amylase (140 μg total protein in 500 μl) were incubated with starch granules as described previously (2.2.8.2). As a control, de-proteinised starch granules were incubated in buffer without protein. Western analysis was carried out using α -amylase antibodies (Fig.18). As in the case of GWD, the binding capacity of the starch surface depended on the dark/light pre-treatment of turions used for the starch isolation. α -amylase did bind the starch granules from dark-adapted turions, but the highest level was measured using starch from pre-irradiated turions (1cR). However, the level of bound α -amylase decreased when granules were isolated from turions pre-irradiated for 96 h with cR (Fig.18/A). In a similar *in vitro* binding experiment, α -amylase from *S. polyrhiza* was replaced by commercially available α -amylase purified from barley (catalogue No. 44E-BARBP; 25 $\text{nmol ml}^{-1} \text{ s}^{-1}$; Gamma-Chemie, München, Germany). The barley enzyme bound to starch granules isolated from dark-kept turions, but the binding capacity was almost lost when starch granules were prepared from turions that had been pre-irradiated for 4 days with cR (data not shown). It has to be mentioned that the transient increase on binding capacity of the commercial α -amylase was not observed.

A





b) Association of β -amylase with starch granules may be artificial and, therefore, without physiological relevance. However, quantification of the binding of β -amylase *in vitro* to starch granules that were prepared from turions before or after the onset of starch breakdown could provide additional evidence for structural changes occurring on the surface of these particles. β -amylase was partially purified from turions, which were irradiated with a single red light pulse and then kept for 3 days in darkness. Starch granules were isolated from turions with the 10-fold volume of the extraction buffer. Using this method, the amount of proteins with weak starch binding capacity was decreased (see 3.1.2). Subsequently, granules were incubated with β -amylase. The Western blot analyses, including controls incubated without β -amylase, are shown in Fig.19A. The control samples (empty columns) contained a small quantity of starch-associated β -amylase. Obviously, the modified starch granule purification procedure does not completely prevent the binding of β -amylase to the granules. The different levels of β -amylase in the various control samples might be caused by the high level of the soluble β -amylase following pulse irradiation (Luka et al., 1999). The *in vitro*-binding of β -amylase was most effective with starch granules obtained after pretreatment of turions with a single Rp (Fig. 19B; hatched columns). Binding, although to a lesser extent, was also observed with granules prepared from darkened turions. In contrast to the Rp, cR (96 h) did not increase the level of the *in vitro*-bound β -amylase. Under these conditions, net starch degradation was initiated. When granules were isolated from turions that had been pre-irradiated with continuous red light for 24 h; 48 h; 72 h and 96 h, the amount of the *in vitro*-bound β -amylase decreased below the limit of detection (Fig.20).

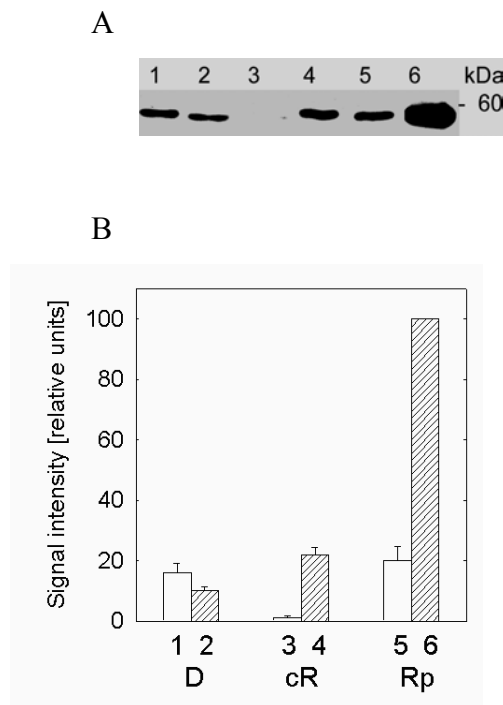
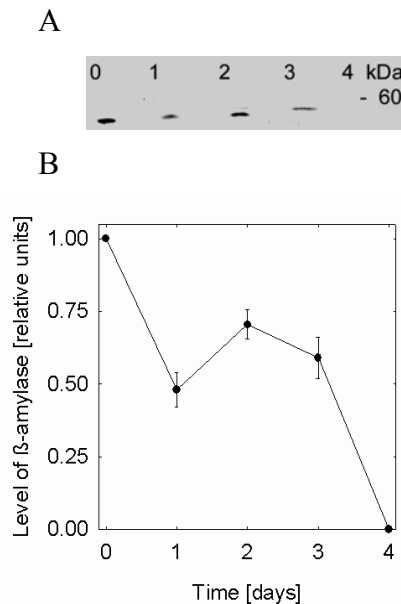


Fig.19: In vitro binding of partially purified β -amylase to starch granules. β -amylase was isolated from turions irradiated with Rp and cultivated for additional 3 days in darkness. Starch granules were isolated from turions after three different light treatments (described below), using the 10-fold volume of the extraction buffer. Following the incubation with β -amylase, the amount of the starch-bound enzyme was determined by Western analysis. A: Representative examples of Western blot signals represented in B. The numbers designate the same samples as below. B: Relative signal intensities of β -amylase binding capacity *in vitro*. Various pre-treatments of turions: cR = 96 h; D = 7 days darkness; Rp: 3 days darkness plus single Rp plus 4 days darkness. Hatched columns: samples incubated with β -amylase. Open columns: controls, incubated without β -amylase.

Fig.20: In vitro binding of partially purified β -amylase to starch granules. A: Representative samples of Western blot signals. The numbers designate the same samples as below. B: Relative signal intensities of β -amylase binding capacity *in vitro* as represented in A). Turions were kept for 3 days in darkness (time zero) and were then irradiated for 4 days with cR. Starch was isolated at daily intervals.



3.3.2. Modifications of starch surface as observed by scanning electron microscopy

SEM was carried out for starch granules isolated from turions kept in darkness (D), or irradiated respectively for 24 h (1cR); 48 h (2cR); 72 h (3cR) and 96 h (4cR) (Fig.21). The surface of starch granules isolated from dark-kept turions was relatively smooth, whereas a certain relief and increased roughness appeared on the granules isolated from irradiated turions. Interestingly, these modifications were also observed on the starch surface of turions irradiated for 24 h (1cR), which is before a significant process of starch degradation took place.

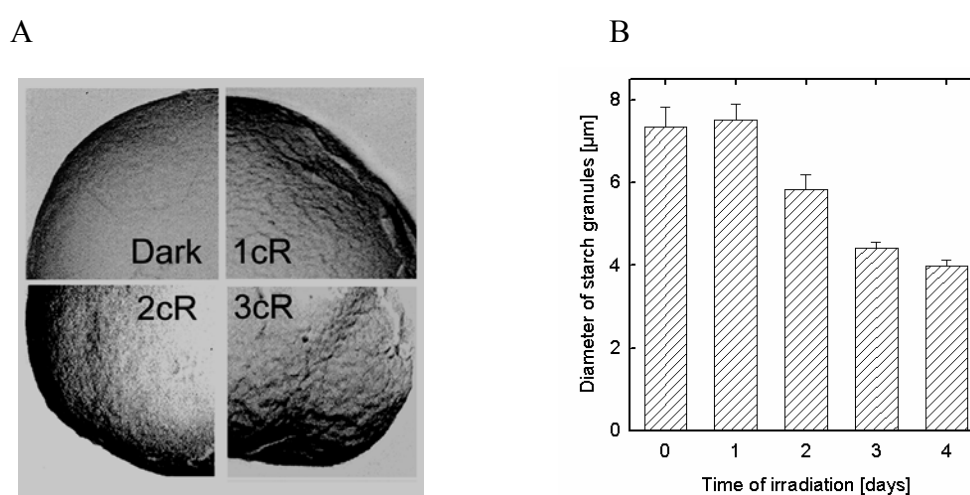


Fig.21: Scanning electron microscopy of starch granules from turions of *S. polyrhiza*. A: Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h (1cR); 48 h (2cR); 72 h (3cR) and 96 h (4cR). B: The diameter of starch granules was measured using scanning electron micrographs. The average of diameter of approximately 50 images for each dark/light pre-treatment of turions was calculated.

3.3.3. Modifications of starch surface as observed by non-contact atomic force microscopy

Structural analysis of the starch surface was performed using nc-AFM. Starch granules isolated from dark-kept turions or turions irradiated for 24 h and 96 h were used. The following modifications were observed for selected granule surfaces: the starch granules revealed the same structure, but some increasing roughness and some surface protrusions were recognized on the irradiated granule surface. (Fig.22/Dark; 1cR and 4cR). A detailed scanning of the surface with the highest possible magnification revealed some randomly organized surface elements. In the dark-kept and irradiated (96 h) samples, two kinds of granula elements were observed: circular (Fig 22/Dark/1 and 22/4cR/1) and oblong

(Fig.22/Dark/2 and 22/4cR/2), whereas only the circular elements were observed in the 24 h irradiated samples (Fig.22/1cR/1). These elements were likely the same, but oriented in a different way on the surface. Observed from the top, they looked like circles, when observed from the side (in a „lying“ position) they were seen oblong. These kinds of elements might be considered as differently tight packed carbohydrate lamellae on the granula surface. Two other observations should be mentioned: 1) The structural elements described above had different sizes depending on the irradiation of turions. The ratio between their dimensions, however, was the same in spite of irradiation (Table 3). This means that the surface elements might be of the same origin and may have grown at the same ratio under irradiation. 2) When granules from dark-kept turions and granules from 24 h irradiated turions were compared, the density of these elements increased respectively from $160/\mu\text{m}^2$ to $250/\mu\text{m}^2$. On the granules isolated from 96 h irradiated turions the density of the elements decreased to $50/\mu\text{m}^2$.

Starch Granules	Short-Circular elements	Long – oblong elements		Ratio Long Diameter/ Short Diameter
	<i>Diameter</i>	<i>Longer Diameter</i>	<i>Shorter Diameter</i>	
D	68 nm	120 nm	50 nm	2.4
1cR	72 nm			
4cR	98 nm	170 nm	70 nm	2.45
Ratio → 4cR/D	1.4	1.4	1.4	

Table 3: *Changes on the dimensions of starch surface elements.* Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h and 96 h were observed under nc-AFM and the dimensions of the respective surface elements were measured.

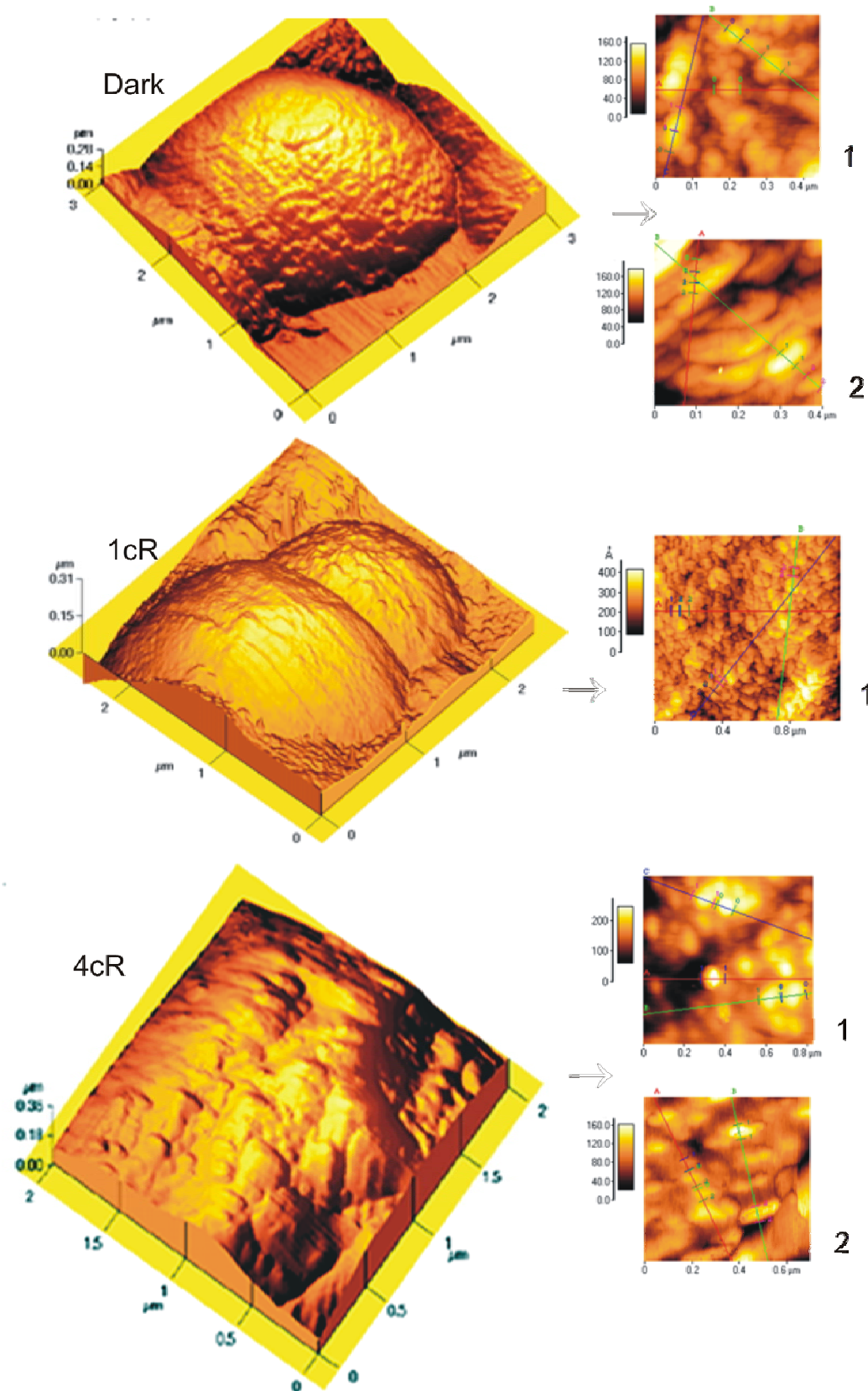


Fig.22: *nc-AFM images of starch granules and the starch surface elements.* Granules were isolated respectively from dark-adapted turions (Dark); irradiated for 24 h (1cR); or irradiated for 96 h (4cR). /1: images of the circular surface elements. /2: images of oblong surface elements

3.4. Influence of phosphorylation (dephosphorylation) on the binding of GWD to starch

3.4.1. Influence of ATP

When isolated starch granules were incubated with [$\beta\gamma$ - ^{33}P]ATP, starch became labelled (Fig. 23). No significant influence of [γ - ^{33}P]ATP was detected demonstrating that only the β -position on phosphate in ATP was used to phosphorylate starch. The level of starch phosphorylation was higher in granules isolated from turions irradiated for 24 h (1 cR) than from dark-kept turions.

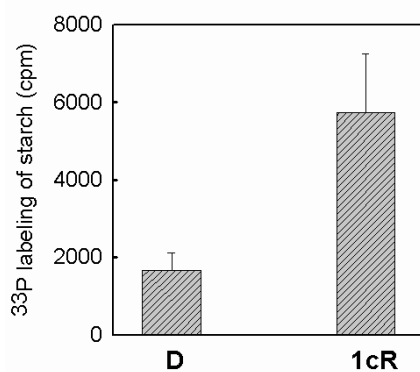


Fig.23: Labelling of starch granules with [$\beta\gamma$ - ^{33}P]ATP. Starch granules were isolated from irradiated turions (1cR) or dark-adapted (D) turions and incubated with enzymatic randomised [$\beta\gamma$ - ^{33}P]ATP. Starch-associated proteins were removed and the granules were thoroughly washed, suspended in water and measured in a scintillation counter. As a control, starch granules from irradiated turions were incubated with [γ - ^{33}P]ATP which resulted in 400 cpm.

In the next experiment, the question was tested whether phosphorylation of starch influences the binding capacity of GWD *in vitro*. Starch granules were isolated from dark-kept turions (D) or turions irradiated for 24 h (1cR) and incubated with ATP. As already mentioned this treatment enhances the degree of phosphorylation of starch, especially in samples from irradiated turions (cf. Fig. 23). Controls were incubated in buffer without ATP. After the incubation, starch-associated proteins were removed with SDS. Soluble proteins were extracted from irradiated turions (1cR) and used for incubation with the de-proteinised starch granules. Thereafter, proteins bound during incubation were extracted, and the amount of GWD was determined by SDS-PAGE and Western analysis. When starch granules were isolated from irradiated turions, the amount of starch-associated GWD was enhanced by ATP treatment (Fig.24). In contrast to starch granules isolated from irradiated turions, those from dark kept turions did not show any effect of ATP treatment on the amount of bound GWD.

A



B

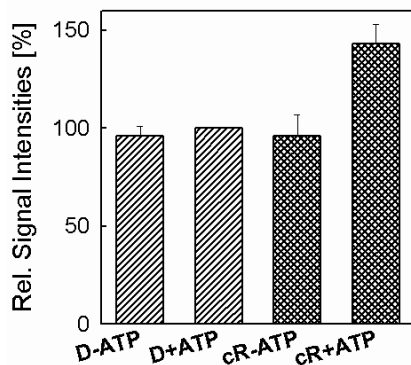


Fig.24: Western blots of GWD after treatment with ATP. Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h (1cR), were preincubated without (-) or with (+) ATP. After this treatment, starch granules were de-proteinised with toluene and incubated with coarse plant extract from 24 h irradiated turions. Then the starch-associated proteins were extracted and separated by SDS-PAGE. A: Western blots of GWD after *in vitro* binding to starch granules. D/Dep and 1cR/Dep: de-proteinised starch granules incubated without soluble protein. D/-ATP and 1cR/-ATP: de-proteinised starch granules pre-incubated without ATP. D/+ATP and 1cR/+ATP: de-proteinised starch granules pre-incubated with ATP. B: Relative intensities (in %) of Western signals represented in A.

3.4.2. Influence of phosphatase

In the next experiment, the question was addressed whether the phosphorylation status of GWD itself has an influence on the capacity of starch binding. As shown before, starch-associated GWD is partially phosphorylated in starch granules from irradiated turions, and the level of phosphorylation can be decreased by *in vivo* treatment with acidic phosphorylase (cf Fig.14/D). Starch granules were isolated from turions irradiated for 24 h (1cR) and incubated in the presence of acid phosphatase. Following centrifugation (5 min, 15,000 g), the relative amount of GWD was determined in the supernatant (released from starch surface and therefore soluble) and in the pellet (starch-associated) by SDS-PAGE and Western analysis (Fig.25A). In controls without phosphatase treatment, GWD was detected exclusively in starch-associated form but not in the supernatant (Fig.25A, lane 1 and 2). During the dephosphorylation by the phosphatase treatment, however, a significant amount of GWD was detected in the supernatant, released from the starch surface (Fig.25A lane 3 and 4). It can be concluded that dephosphorylation of GWD decreased its starch binding capacity.

In another experiment, starch granules isolated from 24 h irradiated turions were treated with alkaline phosphatase (2.2.10.3; Fig.25B). This treatment did not have any influence on the binding of GWD to starch. As in the controls (starch incubated without alkaline phosphatase:

Fig.25B lane 1 and 2), GWD remained associated with starch after phosphatase treatment (Fig.25B lane 3 and lane 4). Preliminary experiments proved that also the 2D pattern of GWD was not affected by alkaline phosphatase treatment (data not shown), i.e. alkaline phosphatase does not dephosphorylate GWD.

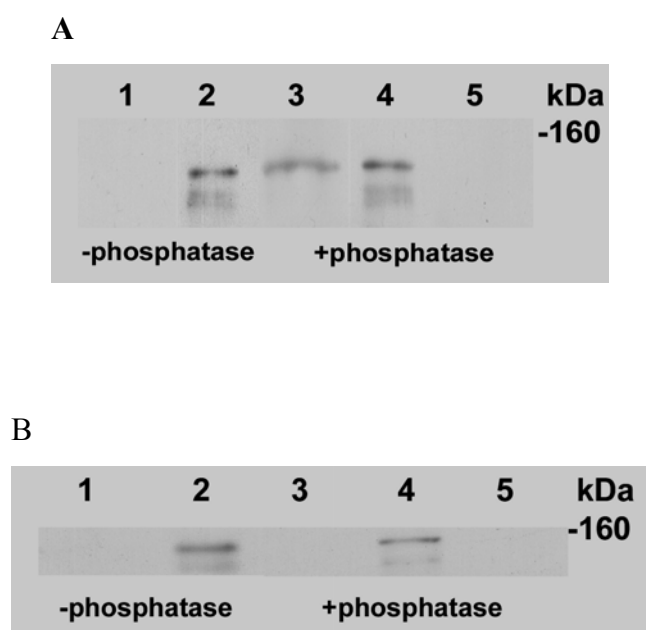


Fig.25: Western blots of GWD protein after treatment with phosphatase. A: Starch granules isolated from irradiated (24 h) turions were incubated without (-phosphatase) or with (+phosphatase) acid phosphatase. Lane 1: soluble GWD (-phosphatase). Lane 2: starch-associated GWD (-phosphatase). Lane 3: soluble GWD (+phosphatase). Lane 4: starch-associated GWD (+phosphatase). Lane 5: only phosphatase. B: Starch isolated from turions irradiated for 24h were incubated without (-phosphatase) or with (+phosphatase) alkaline phosphatase. Lane 1: soluble GWD (-phosphatase). Lane 2: starch-associated GWD (-phosphatase). Lane 3: soluble GWD (+phosphatase). Lane 4: starch-associated GWD (+phosphatase). Lane 5: only phosphatase.

3.5. Analysis of starch degradation *in vitro*

Isolated starch granules were used as an *in vitro* test system not only to investigate binding proteins and processes related to phosphorylation of proteins and starch, but also to investigate the rates of *in vitro* degradation. The role of native starch-bound proteins on the process of starch degradation *in vitro* was analysed in details as follows:

- 1) The „*in vitro* degrading activity“ of starch-associated and soluble proteins from turions of *S. polyrhiza*.
- 2) The influence of ATP and phosphatases treatment on the starch degradation *in vitro*.

3.5.1. *In vitro* degrading activity of starch-associated proteins in turions

The degrading activity of starch-associated proteins was estimated by measuring the amount of soluble carbohydrates released by starch degradation *in vitro*. Soluble carbohydrates were further degraded by acidic hydrolysis and the amount of glucose was measured by a colorimetric method (2.2.9.1). In this experiment, starch granules isolated from turions kept in darkness, or irradiated for 24 h and 48 h were incubated in buffer for different periods in the range of hours (Fig.26). Subsequently, the suspension was centrifuged and the soluble products released from starch were analysed according to the standard procedure. As shown in Fig.26, the starch-associated proteins in turions were able to degrade starch during the incubation time, but the degrading activity was strongly depending on light pre-treatment of turions. After 4 h of incubation, the highest activity was observed in the samples originated from turions irradiated for 24 h. The rate of starch degradation of dark and 48 h irradiated samples were very similar to each other. The amount of released carbohydrates was two times lower than that from 24 h irradiated turions. When the same experiment was carried out using de-proteinised starch granules or commercial potato starch (Sigma) as controls, no degrading activity was detectable (Fig.26/dotted lane), which demonstrates that the release of carbohydrates was caused only by starch-associated proteins.

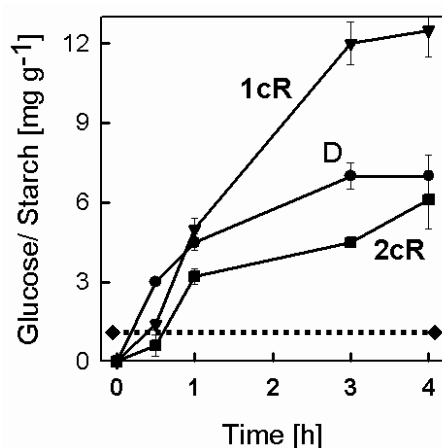


Fig.26: Degradation of starch granules *in vitro*. Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h (1cR) and 48 h (2cR) were incubated in buffer. After separation of soluble products and acidic hydrolysis, glucose was measured according to Waffenschmidt and Jaenicke (1987). Dotted line: de-proteinised and commercial potato starch.

3.5.2. *In vitro* degrading activity of soluble proteins in turions

The role of soluble proteins on the *in vitro* starch degradation in turions was tested for starch granules isolated from turions kept in darkness or irradiated for 24 h. First, starch granules were de-proteinised in order to remove the native associated proteins from the starch surface. Subsequently, the granules were incubated with an extract of soluble proteins from turions irradiated for 24 h. Control samples were incubated in buffer without proteins. During the incubation time, several soluble proteins from turions were bound on the starch granule surface (see here 3.3.1). The *in vitro* degradation assay was carried out, and the *in vitro* degrading activity of the starch-bound proteins was measured. As shown in Fig.27, no effect of light pre-treatment of turions was observed. For both starch samples (isolated from dark-kept and 24 h irradiated turions), the degrading activity was lower than the degrading activity of native starch-associated proteins of the same dark/light pretreated samples.

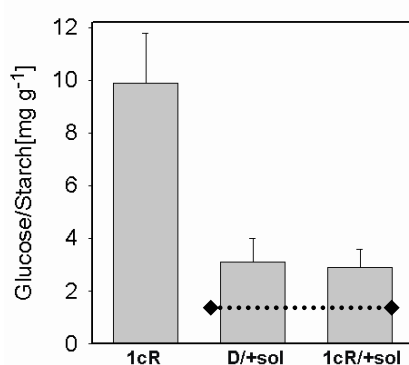


Fig.27: *In vitro* degradation of starch granules incubated with soluble proteins. Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h (1cR) were de-proteinised. Soluble proteins extracted from turions irradiated for 24 h were incubated with the starch granules [(D/+sol); (1cR/+sol)]. Controls were incubated in buffer without proteins (dotted line). After the incubation, the starch degradation assay was carried out and the glucose released was measured according to Waffenschmidt and Jaenicke (1987). For comparison, the amount of glucose released as the result of the activity of starch-associated proteins after 24 h irradiation is given (1cR).

The degrading activity did not change when the de-proteinised starch granules were incubated with soluble proteins plus recombinant GWD from potato, or when starch granules were incubated with recombinant GWD in the presence of native starch-associated proteins from turions (data not shown).

3.5.3. Degrading activity *in vitro* after treatment with ATP and phosphatases

As shown before, ATP and acid phosphatase treatment of starch granules caused modifications of the GWD pattern (see Fig.12 and Fig.14). In the following experiments, the influence of :

- a) ATP and
- b) acid and alkaline phosphatase treatments

on starch degradation *in vitro* were tested.

a) Starch granules isolated from turions kept in darkness or irradiated for 24 h were incubated in ATP assay buffer. Control samples were incubated in buffer without ATP. After the incubation, the degradation products were measured as described above (2.2.9.1). A strong effect of ATP treatment on starch granules isolated from turions irradiated for 24 h was observed. This effect was clearly not detectable with starch granules from dark-adapted turions (Fig.28).

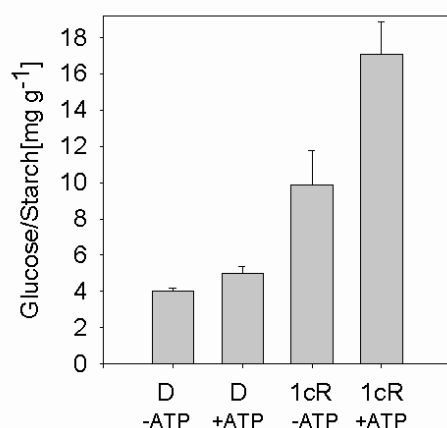


Fig.28: Starch degradation *in vitro* after ATP and acid phosphatase treatment. a) Starch granules isolated from turions kept in darkness (D) or irradiated for 24 h (1cR) were incubated without (-ATP) or with ATP (+ATP). Subsequently, the starch granules were washed and further incubated in starch degradation buffer. After the incubation, the degradation products were separated by centrifugation, and glucose was measured according to Waffenschmidt and Jaenicke (1987).

b) To study the effect of phosphatases on starch degradation *in vitro*, starch granules from irradiated turions (24 h) were pretreated with 1) acid phosphatase and 2) alkaline phosphatase as already described (2.2.10.2 and 2.2.10.4). Control samples were pre-incubated in buffer without phosphatases. Thereafter, the suspension was centrifuged and the starch granules were washed to remove the rest of phosphatases. The standard degradation assay was carried out (2.2.9.1), and the amount of glucose formed (from the released carbohydrates) was

estimated as shown in Fig.29. Neither acid phosphatase, nor alkaline phosphatase treatment did affect the process of starch degradation *in vitro*, because no different rates of starch degradation were detected when controls and pre-treated samples were compared (Fig.29).

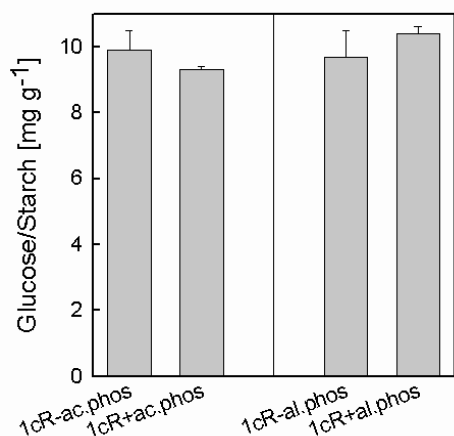


Fig.29: Starch degradation *in vitro* after phosphatases treatment. Starch granules isolated from turions irradiated for 24 h were pre-treated with acid (+ac.phos)/alkaline (+al.phos) phosphatase, or without acid (-ac.phos)/alkaline (-al.phos) phosphatase. Subsequently, the starch granules were washed and further incubated in starch degradation buffer. After the incubation, the degradation products were separated by centrifugation, and glucose was measured according to Waffenschmidt and Jaenicke (1987).

3.5.4. Analysis of *in vitro* degradation products by HPAEC-PAD

A HPAEC-PAD technique was carried out in order to analyse the nature of the starch degradation products *in vitro*. For the analysis, starch granules from turions irradiated for 24 h were used. The sample preparation procedure is described in 2.2.14. The results of this analysis are shown in Fig.30. Carbohydrates with a low molecular weight, like glucose and maltose were detected. Carbohydrates with a polymerisation degree of 3 and 4 were abundant products. However, also carbohydrates with higher degrees of polymerisation (5-8) were detected at a lower concentration. When de-proteinised starch samples were analysed, no carbohydrates were detectable.

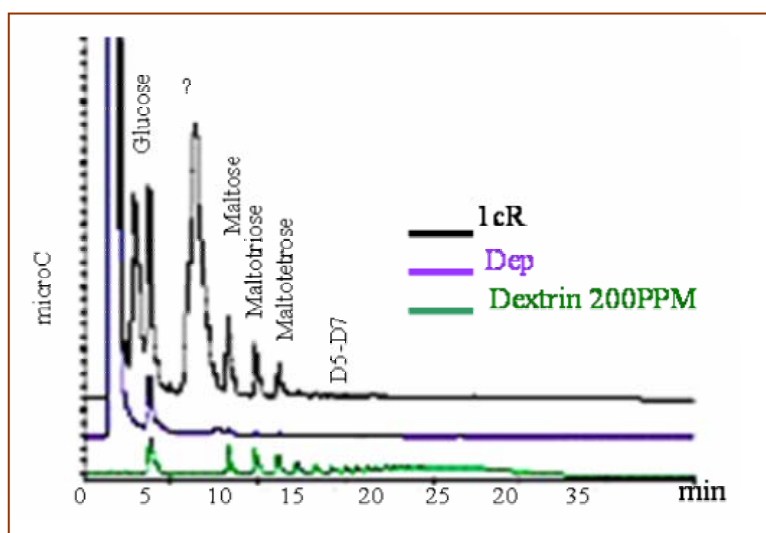


Fig.30: Analysis of starch degradation products *in vitro*. Starch was isolated from turions irradiated for 24 h. Carbohydrates released from degradation *in vitro* of starch granules were determined by HPAEC-PAD analysis. ? = Not identified product.

4. DISCUSSION

In the present work, the interaction between starch granules and proteins associated with the starch surface was studied in order to understand the regulatory mechanisms of starch degradation. Turions of *Spirodela polyrhiza* are a very suitable model system for this study for the following reasons:

1. The degradation of storage starch is well separated from the process of starch synthesis. Whereas storage starch changes are connected to the cycle of seasons (synthesis in summer/autumn, degradation in spring), changes of transitory starch are related to circadian rhythms with overlapping periods of synthesis and degradation. Thus, degradation of storage starch can be investigated without disturbance by simultaneous synthesis in the same cells.
2. Degradation of storage starch in turions can be switched on and off simply by using different light conditions, thus making this process easy to manipulate. Turions represent the only known system in higher plants with light-dependent starch degradation mediated by the photoreceptor phytochrome.
3. Etiolated turions, induced by phosphate-limitation (Appenroth *et al.*, 1996) represent very homogenous populations in which physiological responses are even more uniform by standardised cold after-ripening (5°C) and adaptation to the response temperature (25°C) before light treatment. This property makes it possible to investigate kinetics of several responses very precisely.

It has been shown previously that the rapid degradation of starch in non-dormant turions is mediated by phytochrome (Appenroth and Augsten, 1990; Dölger *et al.*, 1997; Appenroth and Gabrys, 2001) and requires irradiation over an extended period. Under standardised conditions, approximately 90% of the storage starch was degraded within a period of 6 days.

Using these important advantages of turions in analysing starch degradation, an *in vitro* assay system has been developed in which isolated starch granules from differentially (light/dark) pre-treated turions were used to investigate possible

phosphorylation of starch-bound proteins, phosphorylation of starch surface, binding of proteins to starch surface and degradation of starch *in vitro*. This *in vitro* test system makes it possible to relate signals inducing starch degradation *in vitro* to early steps of this complex process.

4.1. Characterization of starch-associated proteins during starch degradation in turions

In the present work, the hypothesis has been tested that in the process of degradation of storage starch association of proteins with the starch granule surface represent an essential regulatory step. Following the concept of Morrison and Karkalas (1990) and of Mu-Forster *et al.* (1996), proteins bound to the surface of a starch particle (starch-associated proteins) are distinguished from those trapped within the particle (internalised proteins) on the basis of different solubilisation procedures. In turions, the starch-degrading enzymes so far investigated exist in three different states: 1) soluble, 2) associated with the starch surface, and 3) enclosed inside the starch granules. Studies on the soluble fraction of starch degrading enzymes indicated that the activities of starch-degrading enzymes such as starch-phosphorylase, β -amylase and α -amylase did not change significantly in accordance with the rate of starch degradation especially in the period before starch degradation could be detected (Luka *et al.*, 1999). Hence, it has been suggested that this fraction (apparently) does not play any physiological role during starch degradation. Using antibodies against GWD and α -amylase, it was demonstrated that these two enzymes were abundant in the soluble fraction. Only small amounts of GWD and α -amylase were associated with starch, and both proteins were not detectable inside the starch matrix. However, assuming that the compartmentation of proteins restricted to the interface of the insoluble starch particle and to the surrounding stroma is related to their function, the selective binding of proteins to the starch granule surface was further analysed. For the first time, the 2D-E images of starch-associated protein fraction demonstrated its complexity (cf. Fig.5). With this proteomic approach that is in progress, a broader analysis of the starch-related proteins might contribute to reveal the mechanisms of starch breakdown.

The complexity of the starch-associated fraction, however, might have been overestimated, since artificial binding of proteins to starch, during and following

homogenization of the plant tissue is possible. This situation is also known in other systems. Studies on isolated starch granules from maize endosperm have shown that the binding of δ -zein to starch surface was artificial since this enzyme was not localised in amyloplasts (Mu-Forster and Wassermann, 1998). The same fact has been reported for the cytosolic phosphorylase which, as a result of a compartmentation loss during homogenisation, bound to the granule surface in potato leaves (Ritte, 1999). To solve this experimental problem, turions were homogenised using a higher volume of buffer than normally applied (10-fold or 100-fold). In this way, two possible effects were expected: First, binding of those proteins that artificially attach to the starch granules would decrease. Second, weakly-bound proteins were expected to be lost regardless of their original (i.e. *in vivo*) compartmentation. By using a larger volume of extraction buffer, a low temperature (5°C) and a short period of time for starch granule preparation (in the range of a few minutes), proteins bound to the starch granules *in vivo* should have been preferentially isolated, whereas artificial binding should have been less significant. As shown in Fig.6, the modified starch isolation procedure did not change the level of bound GWD and that of α -amylase, but clearly lowered the level of bound β -amylase as well as the level of two isoforms of α -glycosidase.

Therefore, to initiate starch degradation, the binding of GWD and α -amylase was considered to be more relevant than that of β -amylase. The studies on a wide range of starch accumulating species including sweet potato and yam, seeds of maize and barley, banana fruit (Ritte *et al.*, 2000a), as well as on the *sex1* mutant (defective in GWD) in *Arabidopsis* (Yu *et al.*, 2001), suggested that GWD might have a general function in the plant kingdom and the question for the physiological relevance of GWD binding must be addressed. The crucial role of endoamylases for the initiation of starch breakdown has also been previously reported (Smith *et al.*, 2003, Witt and Sauter, 1996; Witt and Sauter, 1995). Therefore, the selective binding of GWD and α -amylase to the starch granules was studied here in detail in close relation to starch degradation in turions.

In non-dormant turions, the amount of starch-associated GWD declined rapidly once initiation has occurred and GWD was gradually released from the starch particles. It

therefore appears that binding of GWD to the starch granule surface is a pre-requisite for the starch initiation of starch breakdown but did not *per se* initiate the degradation. When the turions were irradiated in a nutrient solution lacking phosphate, starch degradation was impeded and the level of starch-associated GWD remained high. In dormant turions, the level of starch-bound GWD increased (and did not decrease) with time and the starch content did not change. It should be mentioned that the level of starch-binding proteins was estimated on the basis of the dry weight of starch. It is likely that during starch degradation the surface-to-mass ratio of the particle population increased. Therefore, the data shown in Fig.8 even underestimated the changes in the content of GWD for individual starch particles.

The interaction of GWD with starch in turions as presented in this work differed from that reported for leaves. In potato and pea leaves, GWD was found to occur in a starch-associated form during the dark period (when starch was degraded) but was released during the light period when net starch synthesis took place (Ritte *et al.*, 2000b). In tobacco leaves, GWD bound to starch was clearly detectable at the end of the light period, but its amount strongly increased after three hours of darkness (Ritte, Steup and Häusler, unpublished cited in Reimann *et al.*, 2002). Whereas transitory starch is mobilized in darkness, storage starch degradation in turions is induced by light (Appenroth and Gabrys, 2001; Appenroth and Gabrys, 2003). This differing response is caused by the different functions of transitory and storage starch, i.e. the supply of carbon skeleton and energy equivalents during the period without photosynthesis (end of the light period), and at the beginning of a new life cycle (induced by light via phytochrome), respectively. If the association of GWD and α -amylase plays a general role in the degradation of starch, the influence of light on the degree of association is expected to be different in transitory starch and storage starch. This seems precisely to be the case. Moreover, unlike in turions, in all experiments with transitory starch, GWD was bound to granules throughout the entire period of net degradation (i.e. during the whole dark period) and the decrease observed in turions was not found in transitory starch. However, it is possible that in leaf cells, due to the higher morphological complexity of the organ, the initiation of net starch degradation is not very well synchronised. If so, starch isolated from dark-adapted leaves could comprise granules originating from plastids at various stages of starch degradation, ranging from a pre-initiation state to an advanced mobilisation. In

this case, it cannot be excluded that the GWD present in the total starch fraction was derived from a distinct particles subpopulation. In the turions system, the situation is much clearer, as already mentioned above.

The binding of α -amylase to starch granules differed from that of GWD in several aspects:

- 1) The onset of irradiation with cR was accompanied by a significant (although transient) increase in the amount of bound α -amylase.
- 2) Once starch degradation was initiated, the hydrolase was released more rapidly from the granules than GWD.

As in the case of GWD, phosphate deficiency which prevented starch degradation also prevented the dissociation of α -amylase from the starch granules. These data lead to the conclusion that α -amylase in turions is necessary only during the early phase of degradation of storage starch.

The selective binding of GWD and α -amylase to the starch surface is due to a direct protein-carbohydrate interaction without other proteins being involved. Two experimental evidences support this conclusion: First, *in vitro* binding experiments, the binding of both GWD and α -amylase occurred as well after de-proteinisation of the starch surface by toluene or SDS. Secondly, the level of bound GWD and α -amylase decreased after *in vitro* treatment of starch granules with maltodextrine. Starch-degrading products like maltose did not affect the binding of the two enzymes to starch. The release of both enzymes was the result of a competition between starch and starch-like molecules for carbohydrates binding sites of the proteins, also described for endoamylases in poplar wood (Witt *et al.*, 1995b).

4.2. Regulation of starch-binding capacity by phosorylation via GWD

The observed correlation between the rate of starch degradation and the levels of starch-associated GWD and α -amylase could be attributed either to post-translational modifications of proteins, and/or to alterations occurring at the surface of the starch particle. In this work, 2D-E/ Western blot techniques were used to address the question of protein modifications during starch degradation. These analyses focused

on the effect of light, especially during the initial steps of starch degradation: induction of starch degradation by application of cR for 24 h. Investigations of the starch-associated fraction of GWD from irradiated turions demonstrated the appearance of additional immunochemical signals in the acidic region of gels. These signals were absent in the controls of dark-kept turions where no starch degradation occurred. By combining these data, the conclusion arises that one or several phosphorylated forms of GWD were formed under irradiation. The reasons are as follows: 1) Antibodies raised against GWD from potato recognized the related proteins from turions and the molecular weight was in the same range as that of GWD from other plant species (Ritte *et al.*, 2000a). 2) The concentration of the two proteins in the more acidic region of the gel was increased by incubating isolated starch granules with ATP. 3) The same two proteins which were recognized by GWD antibodies were labelled by incubating isolated starch granules with [^{33}P]ATP by a reaction which uses specifically β -ATP, whereas γ -ATP had no effect. 4) Phosphorylation of starch proceeded during incubation of isolated starch granules with β -ATP, whereas gamma-ATP had no effect in this process. 5) The treatment with acid phosphatase converted the pattern of light-dependent GWD into a form similar to the darkened one (i.e. not phosphorylated form). All of these results reflected the properties of GWD as recently revealed by biochemical investigations (Ritte *et al.*, 2002; Ritte *et al.*, 2003). It can be concluded that the same light signal which induces starch degradation in turions, produces a distinct post-translational modification of GWD consisting in protein phosphorylation. To the best of our knowledge, this light-dependent phosphorylation of starch-associated GWD is shown here for the first time.

Originally, it has been assumed that the shift into the acidic region of the 2D-E described above was directly caused by phosphorylation of GWD. The calculations demonstrated that the expected changes of proteins similar to GWD from *S.polyrhiza* would be too small to account for the observed effects. Whereas it is clear that phosphorylation is involved, other factors must contribute to the unexpectedly large shift of the isoelectric points following irradiation or ATP treatment.

The following experimental results indicated that the properties of soluble GWD contrast to those of starch-associated GWD. First, the 2D pattern of the soluble

protein was not modified when turions were irradiated. Secondly, the ATP treatment of the protein extract from turions failed to produce the phosphorylated form of GWD as shown by 2D-E. Also, efforts to demonstrate the activity of soluble GWD were not successful. One reason for this might have been the presence of protein phosphorylase activity in the plant extract. Therefore, the possible phosphorylation of purified recombinant GWD was also investigated using 2D-E/ Western analysis. The labelling of recombinant GWD by [β - ^{33}P]ATP was demonstrated by Ritte *et al.* (2002) and Ritte *et al.* (2003). However, after incubation with ATP, only very weak signals at the acidic region were detected by 2D-E/ Western analysis. These results demonstrated that only a small fraction of soluble GWD was phosphorylated which, most probably, is the reason for the low sensitivity of the activity-assay (Ritte *et al.*, 2003). It can be concluded that an effective phosphorylation of GWD requires the association of this protein with starch.

Binding studies using the *in vitro* assay system clearly demonstrated that there are changes on the starch surface which control the selective binding of proteins during starch degradation. The most remarkable fact of this series of experiments is that during degradation, starch granules lost the capacity to bind soluble, partially purified GWD, α - and β -amylase from turions. The same holds true when commercially available α - and β -amylase were incubated with isolated starch granules from dark-adapted and irradiated turions. It has to be mentioned that the transient increase in binding level was observed only in the case of soluble α -amylase and not when commercial α -amylase was used. This indicates that this transient increase is specific for endogenous amylase from turions. The hypothesis that alterations of the starch surface were involved in the reversible binding of proteins is also in agreement with binding experiments using recombinant GWD and isolated potato leaves starch granules (Ritte *et al.*, 2000b). Recombinant GWD bond to starch granules isolated from illuminated or darkened leaves and the binding to the latter was more effective. Taking together, these results could be interpreted as follows:

- 1) The starch-binding sites are modified during starch degradation.
- 2) The modification of the starch surface through phosphorylation influences the starch-binding sites.

This interpretation fits well with the analysis of starch surfaces using scanning electron microscopy and non-contact Atomic Force Microscopy. The transient increase of the density of structural surface elements and the increased roughness after 24 h irradiation, followed by a reduced density of the structural elements subsequent to light application, may reflect alterations of the starch surface during starch breakdown. The structural elements became larger most likely due to enhanced binding of water molecules to the carbohydrate helices. It could also be that the surface carbohydrate helices are bent into superhelices by new inter-carbohydrate hydrogen bonds. Such a process involving surface OH-groups might result in an altered binding capacity of granule surface towards proteins.

The described alterations of the starch surface, as observed by SEM and nc-AFM, implicates the role of GWD in phosphorylating starch after light application. The enhanced *in vitro* phosphorylation of starch from turions after 24 h irradiation and the biochemical properties of GWD (Ritte *et al.*, 2002) support this hypothesis. However, it cannot be excluded that the phosphorylation of GWD itself influences the binding capacity of this protein to starch granules. The treatment of starch granules taken from irradiated turions using acid phosphatase diminished the GWD phosphorylation level, and decreased the binding capacity to starch granules. It can be concluded that GWD phosphorylation *in vitro* enhanced its binding to the starch surface. Irradiation of turions also resulted in phosphorylation of starch *in vitro*. Following starch de-proteinisation, the binding capacity of soluble (i.e. non-phosphorylated) GWD increased. This effect was even more pronounced when starch granules from irradiated turions were pre-incubated with ATP. It is therefore evident that phosphorylated starch binds more GWD *in vitro*. However, the effect of enhanced binding by treatment of starch granules with ATP seems to be without physiological relevance because no increased level of GWD following irradiation could be detected *in vivo*.

Thus, the data presented here suggest that the regulation of the reversible binding of proteins on the starch surface depends upon the light-induced phosphorylation of starch. GWD acts as a starch-water-dikinase, making starch more accessible to degradation. The degree of starch phosphorylation controls the binding of other proteins like α -amylase. Presumably, this was the reason why after 24 h irradiation

more α -amylase bound to the surface of starch granules *in vitro* and *in vivo*. Therefore, the regulation of the starch-binding capacity via phosphorylation of starch mediated by GWD represents a regulatory step in the starch degradation process.

Finally, it should be mentioned that Rp had a very significant effect on the binding capacity of β -amylase as shown in Fig.19. Although no starch degradation was induced under this condition, the pulse dramatically enhanced the binding capacity of β -amylase to starch. Preliminary experiments with GWD and α -amylase, however, have shown that the levels of starch-associated GWD and α -amylase have not changed in the periods of 12 h, 24 h, and 48 h after Rp (data not shown). Experiments with shorter dark periods following Rp application should be carried out in the future. It seems that the triggering effect of a light pulse represents a very early event in the starch breakdown process whose chemical nature remains to be revealed.

4.3. Regulation of starch degradation by phosphorylation of starch

The next central question addressed here is how and to which extent the starch phosphorylation process controls starch breakdown. The relationship between starch degradation and the starch-phosphate content was not obvious when starches from different species were compared (Ritte *et al.*, 2000a). In the *sex1* mutants and the GWD transgenic potato plants, however, low starch-phosphate content in leaves (~0-1 nmole glucose-6-phosphate per mg starch) was linked to impaired starch degradation (Lorberth *et al.*, 1998; Ritte, 1999; Yu *et al.*, 2001). By contrast, similar phosphate contents in the cereal endosperm apparently did not result in reduced starch degradation (Blennow *et al.*, 2002). Within the frame of this work, despite repeated efforts it was not possible to directly measure the phosphate contents of starch in turions. Obviously, the enzymatic assay adapted for potato leaves (Ritte, 1999) was not sensitive enough in the case of turions. In order to find a possible correlation between the degree of starch breakdown and phosphorylation process, other methods need to be applied. At the moment, experiments are in progress to measure the phosphate content by acid digestion of starch followed by atom fluorescence spectroscopy.

In dark-adapted isolated granules of turions, starch was labelled by β -ATP at lower rates than for the irradiated samples demonstrating increased ability to become phosphorylated under the influence of light. The irradiated samples also showed higher rates of starch degradation, both *in vivo* and *in vitro*. It can be concluded that the activity of associated GWD is triggered by light, and that this in turn promotes starch phosphorylation and starch degradation.

In this work, the newly developed *in vitro* assay was also used to measure the rate of starch degradation in turions. The importance of this system lies in the fact that only those proteins which are bound to the granule surface can initiate the breakdown of starch *in vitro*. The following experimental evidences support this finding: First, the rate of starch degradation of de-proteinised starch granules is below the detection limit. Second, no starch degradation products were detected from the deproteinised starch granules using HPAEC-PAD analysis. Third, the degradation rate of commercially available potato starch was under the limit of detection. The *in vitro* test system demonstrated that the granules isolated from turions irradiated for 24 h showed the highest rate of starch degradation. Moreover, the rate of starch degradation was further enhanced by treating isolated starch granules (from turions irradiated for 24 h) with ATP. Interestingly, there was no influence of acid phosphorylase on the rate of starch degradation *in vitro*. This result seems to be in contrast to the de-phosphorylating effect of acid phosphorylase on GWD. The reason is that the ATP effect on starch degradation was mediated by starch phosphorylation and only as an intermediate step by GWD phosphorylation. Acid phosphatase dephosphorylated GWD, but this enzyme did not remove the starch-bound phosphate (Ritte, personal communication). Also, the degree of starch degradation was not affected by alkaline phosphatase treatment. The mechanism is different: this enzyme did not de-phosphorylate GWD (data not shown). Therefore, the decisive factor for the regulation of starch degradation is the level of starch phosphorylation, not the phosphorylation of any other protein.

Using the *in vitro* assay system, it was shown that the rate of starch degradation *in vitro* correlated very well with the levels of starch-associated α -amylase *in vivo*. This fact, which was first demonstrated in this study, implies the significant role α -amylase

plays in the initiation process of starch degradation *in vivo* as previously suggested by Smith *et al.* (2003).

Taking all of the presented data into account, the sequence of events ultimately leading to starch degradation involves the following steps as shown in the working model of Fig.31.

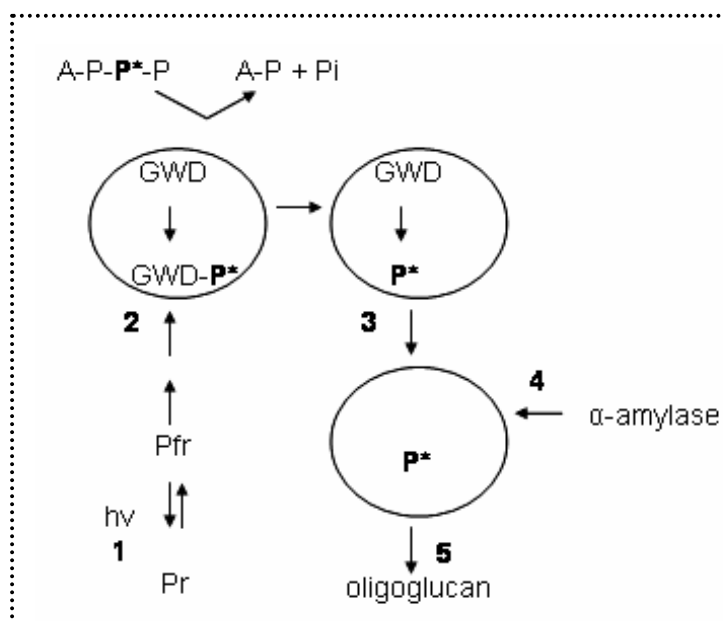


Fig.31: Scheme representing the sequential events of light-induced degradation of storage starch in *turions*. **1) Phototransformation of phytochrome from its red light absorbing form (Pr) in its far-red absorbing physiologically active (Pfr) form which starts a signal transduction chain.** **2) Activation of starch-associated kinase GWD and phosphorylation of GWD using β -phosphate group of ATP.** **3) Phosphorylation of starch.** **4) Enhanced association of enzymes like α -amylase.** **5) Initiation of starch degradation and release of oligoglucans from starch granules.**

5. THESIS

1. The degradation of storage starch in turions, survival organs of *Spirodela polyrhiza*, is induced by light and mediated via phytochrome. Protein-starch interactions that occur during starch degradation were used as a tool to investigate initial events in the regulation of starch degradation. Two fractions of starch-related proteins were distinguished: the starch-associated proteins attached to the granule surface and the starch-internalized proteins that are enclosed within starch particles. The pattern of starch-associated proteins was more complex than that of internalised proteins and varied depending on the metabolic state of the turions. Following different dark/light pre-treatments of turions, proteins bound to the starch granule surface were analysed by SDS-PAGE and, for the first time, by two-dimensional gel electrophoresis (2D-E). During preparation, several proteins became artificially associated with starch (β -amylase, two isoforms of α -glucosydase). In contrast, α -amylase and glucan-water-dikinase (GWD) belong to the spectrum of native starch-associated proteins.

2. Within the first 24h of irradiation (induction of starch degradation in turions), the level of starch-associated α -amylase transiently increased and then rapidly decreased. The amount of associated GWD decreased without a transient increase during irradiation. The dissociation of both α -amylase and GWD from the starch granules precedes the start of net starch degradation in turions.

3. Dark-adapted turions do not undergo starch degradation. Under this condition, α -amylase and GWD were bound to the surface of starch granules and remained unchanged over the time. A similar situation exists under other conditions of no starch degradation (phosphate deficiency, dormant turions).

4. In the granules from irradiated turions (24h irradiation) analysed by 2D-E/ Western blots, additional spots of GWD were detected which were absent in the immunoblots from dark-adapted plants. These spots showed an increased signal intensity when the starch granules were incubated with ATP, became labelled by [$\beta\gamma$ - ^{33}P]ATP, but not by [γ - ^{33}P]ATP, and were removed by treatment with acid phosphatase. This strongly suggests that they represent (a) phosphorylated form(s) of GWD. Thus, it was demonstrated here for the first time that post-translational phosphorylation of GWD *in*

vivo is induced by the same light signal that induces also starch degradation. Analysis of α -amylase indicated that this enzyme was not modified by light. Instead, changes of the starch surface control the reversible binding of this enzyme to the starch surface during the first 24h of irradiation.

5. *In vitro* incorporation of [β - ^{33}P]ATP into starch was increased in turions irradiated for 24h. It is postulated that phosphorylation of starch modifies the surface properties of starch granules that are relevant for protein binding. These modifications of the starch surface after different periods of light treatment were detected by differential binding of several proteins *in vitro* and confirmed by electron microscopy and non-contact atomic force microscopy. The density of the specific elements as detected by atomic force microscopy increased by irradiation before the onset of starch degradation, and thereafter, decreased. These elements may represent the protein-binding sites on the starch surface, because their density changes parallel to the detected amount of starch-associated α -amylase. Thus, increased starch phosphorylation caused increased binding of enzymes like α -amylase.

6. Compared to dark-kept turions, light-induced phosphorylation of GWD has several consequences as indicated by the following results: 1) enhanced (decreased) binding capacity of GWD by ATP (phosphatase) treatment. 2) enhanced incorporation of the β -phosphate group of ATP into starch. 3) increased rate of starch degradation that was further enhanced by phosphorylation of starch.

7. A model has been developed which includes the detected sequence of events from light signaling to starch degradation: (1.) Absorption of light by phytochrome, which activates GWD. (2.) Starch-associated GWD becomes post-translationally modified by autophosphorylation in agreement with the recently discovered biochemical properties of this enzyme. (3.) Starch becomes phosphorylated which (4.) enhances the binding capacity of enzymes as already shown for α -amylase. Finally (5.), hydrolysis of starch is initiated. This model indicates that phosphorylation of GWD is a key event in the regulation of starch degradation in turions.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Es wurde weder die Hilfe eines Promotionsberaters noch die Hilfe Dritter, nicht in dieser Arbeit erwähnten Personen, in Anspruch genommen. Diese Arbeit wurde weder in dieser noch in ähnlicher Form bei einer anderen Hochschule als Dissertation oder Prüfungsarbeit eingereicht.

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